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# THE ANAEROBIC ASSIMILATION OF GLUCOSE BY YEAST CELLS

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For many years it was generally assumed that yeast cells could convert sugars into cellular polysaccharides only in the presence of molecular oxygen. This assumption was based on the fact that sugar is converted quantitatively into fermentation products by yeast cells under strictly anaerobic conditions if the reaction is allowed to continue for an extended length of time. In industrial fermentations, Joslyn (1) has pointed out that 95 per cent of the sugar present is converted to carbon dioxide and alcohol, while the remaining 5 per cent can be accounted for by other products such as glycerol, fusel oil, succinic acid, and lactic acid. Kluyver (2) showed that glucose can be determined with a fair degree of accuracy by measuring the amount of carbon dioxide produced when a small amount of sugar is fermented by a relatively large quantity of yeast. Evidence has accumulated, however, showing that assimilation actually does occur during fermentation and that the quantitative results noted above may be due to the utilization of the stored carbohydrate after the exhaustion of the sugar.

Winzler and Baumberger (3) showed that the heat produced during the fermentation of glucose by a strain of bakers' yeast was only 63.2 per cent of the theoretical value, although the glucose had completely disappeared. From this they concluded that 29.5 per cent of the sugar was assimilated and 70.5 per cent was fermented. Van Niel and Anderson (4) found that only 70 per cent of the theoretical amount of carbon dioxide had been produced when the glucose had completely disappeared. They also showed that much of the remaining 30 per cent could be accounted for by an increased dry weight of the cells. Stier (5) came to the surprising conclusion that a larger fraction of the added glucose was assimilated under anaerobic than under aerobic conditions, since the total hydrolyzable polysaccharide content of the cells increased to a slightly greater extent in the absence of oxygen. Assimilation also occurs during the fermentation brought about by brewers' yeast, since Meyerhof and Schulz (6) found that only 74 per cent of the theoretical amount of carbon dioxide was produced, while the total carbon content of the cells increased to an extent equivalent to the assimilation of about 18 per cent of the added sugar.



The purpose of this paper is to describe experiments in which the carbon dioxide production, the total polysaccharide content, and the glucose concentration were measured at intervals during the anaerobic fermentation of glucose by a suspension of *Saccharomyces cerevisiae*, thereby giving the relationships between the amount of glucose consumed, fermented,<sup>1</sup> and assimilated during the entire course of the fermentations

## EXPERIMENTAL

### Methods

Fresh Fleischmann's bakers' yeast (*Saccharomyces cerevisiae*) was washed three times and suspended in a 0.1 M  $\text{NaH}_2\text{PO}_4$  solution. The fermentations were carried out in a liter Erlenmeyer flask with 500 ml of the yeast suspension. The flask was in a water bath and the suspension was very rapidly stirred by a large glass-covered, motor-driven, magnetic stirrer for the duration of the experiments. The flask had two outlets, one below the surface of the suspension for removing samples and the other above the surface of the suspension. The latter outlet was connected to a 2-way stop-cock, one branch of which led to a gas burette for measuring the carbon dioxide production, while the other branch led to a gas burette filled with nitrogen for replacing the samples that were removed. Before adding glucose, the atmosphere was displaced by nitrogen and the system was allowed to come to equilibrium. After the glucose was added, the carbon dioxide production was measured in the gas burette, care being taken that the system was at atmospheric pressure at all times. The temperature of the water bath, the temperature of the gas burette, and the atmospheric pressure were also recorded. It was assumed that equilibrium was maintained between the gaseous and dissolved carbon dioxide. Thus the total carbon dioxide production was taken as the sum of the observed burette reading and the calculated carbon dioxide in solution. Since the suspension medium was acidic, any variance in the carbon dioxide retention was reduced to a minimum.

Samples of the suspension were withdrawn at intervals during the fermentations and appropriate collections were made for the resulting changes of volume. The following determinations were made on the samples: (1) the reducing value of the cell suspension, (2) the reducing value of the super-

<sup>1</sup> Assuming equal molar concentrations of carbon dioxide and ethanol throughout the fermentations, the carbon dioxide determination becomes a measure of the formation of both products. This assumption seems justifiable, since it was found that little or no acetaldehyde accumulates during the fermentations, all the acetaldehyde formed by the decarboxylation of pyruvic acid being converted to ethanol. Also other investigators (4, 6) found that equal molar quantities were formed in their fermentation studies.

natant fluid after centrifugation, (3) the reducing value of the cells after hydrolyzing for 2 hours at  $100^{\circ}$  with 1 N HCl and neutralizing with NaOH

It can be seen that determination (2) = glucose concentration,  $(1-2)$  = reducing substances in the cells,  $(3-(1-2))$  = "total hydrolyzable polysaccharides"<sup>12</sup>

All of the reducing values were determined as glucose by the Shaffer (8) ferricyanide electrode method. The effect of the electrolyte differences in the samples was overcome by using corresponding concentrations of electrolytes in the reference electrode.

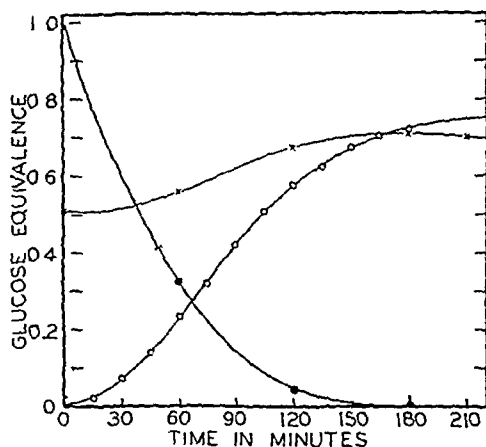


FIG 1 The anaerobic fermentation of 500 mg of anhydrous glucose by a fresh suspension of bakers' yeast. The suspension contained 2.5 gm (wet weight) of yeast made up to 500 ml with 0.1 M  $\text{NaH}_2\text{PO}_4$  solution. ●, glucose concentration, mg per ml, ○, glucose fermented to carbon dioxide and ethanol, mg per ml, ×, total hydrolyzable polysaccharide content of the yeast cells as glucose, mg per ml, the dotted curve represents the intermediary products, estimated by difference.

A stained preparation of the suspension was examined at the conclusion of each experiment. No gross bacterial contamination was ever found.

### Results

The results obtained by the fermentation of 500 mg of anhydrous D-glucose with a freshly prepared yeast suspension are shown in Fig 1.

- It should be noted that the values obtained for the "total hydrolyzable polysaccharide" by this method do not actually represent the total polysaccharides of the cells, since there is an insoluble carbohydrate fraction which is not hydrolyzed by hot hydrochloric acid (7), and ferricyanide is reduced to some extent by other substances present besides the sugars. However, if we assume that these factors remain constant during the fermentation, the increase in the "total hydrolyzable polysaccharide" should give a good approximation of the carbohydrate assimilated.

For convenience, the products are plotted on the basis of their glucose equivalence, *i e*, the glucose utilized in their formation. The glucose consumption curve shows that the sugar had completely disappeared after 180 minutes<sup>3</sup>. At that time 0.72 of a glucose equivalent had been converted into carbon dioxide and ethanol, and the hydrolyzable polysaccharide content of the cells had increased from 0.51 to 0.71 of a glucose equivalent, while 1.00 equivalent of glucose had been utilized. Therefore, 72 per cent of the sugar was fermented to carbon dioxide and ethanol, 20 per cent was assimilated, and 8 per cent formed other products (probably glycerol, succinic acid, etc.). Results of similar experiments are shown in Table I. The extent of fermentation found by the method used in these experiments

TABLE I  
*Anaerobic Fermentation and Assimilation of Glucose by Commercial Strain of Baker's Yeast*

Experiment No	Fate of added sugar		
	Carbon dioxide and ethanol	Synthesized polysaccharides	Other products
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	72	20	8
2	69	20	11
3	71	21	8
4	69	24	7
*	70.5		
†	68.9-72.3		

\* Calorimetric determination by Winzler and Baumberger (3)

† Warburg determinations by Van Niel and Anderson (1)

is in excellent agreement with the results obtained by other methods (see Table I).

From the typical experiment shown in Fig. 1, it can be seen that during the earlier period of the fermentation the glucose was being utilized at a faster rate than the products were appearing, while in the later period the reverse was occurring. In other words the fractions we analyzed failed to account for all of the glucose used by the cells and therefore some of the glucose must be considered to be in an unanalyzed form. This unanalyzed form is called an intermediate in the rest of the discussion. In order to account for the rapid initial decrease in glucose we assume an initially high

<sup>3</sup> A correction was made for a small residue of reducing material remaining in the supernatant fluid at the conclusion of the fermentations. This material was not glucose or accumulated aldehydes, but was made up of other substance exuded from the cells. Yeast cells were unable to ferment any part of this reducing material, even after it had been concentrated 50 times by evaporation.

rate of conversion to intermediates, and similarly the later more complete accountability of the sugar in our analyses we ascribe to a diminution in the quantity of intermediates. The course of the accumulation of intermediary products can be seen in Fig 1, the dotted curve representing the glucose that had disappeared but was not accounted for by the formation of the two main fermentation products or by assimilation. It can be seen that as much as 40 per cent of the added sugar appeared in this curve at one stage of the fermentation.

The heat production during fermentation as measured by the micro calorimeter of this laboratory (3) is shown in Fig 2. Since the heat production curve shows an induction period entirely similar to the carbon dioxide production curve (Fig 1), it is apparent that little or no heat is liberated in the

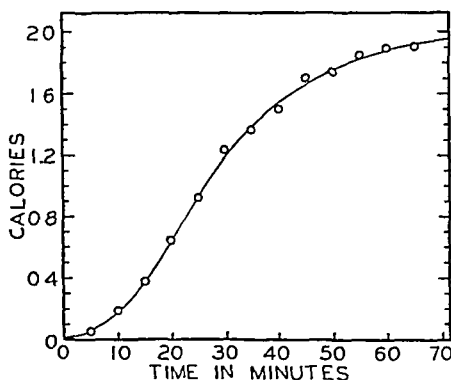


FIG 2 The heat production during the anaerobic fermentation of 25 mg of anhydrous glucose by a fresh yeast suspension. The suspension contained 0.30 gm (wet weight) of yeast, obtained from a 24 hour pure culture of *Saccharomyces cerevisiae*, race FF-17, washed and made up to 20 ml with phosphate buffer (pH 6.8).

formation of the intermediary products during the rapid initial disappearance of glucose. No conclusion can be drawn as to the steps involved in the formation of intermediary products from the calorimetric data at hand, however, since the conversion of acetaldehyde to ethanol (the last step in the fermentation) is the exothermic reaction largely responsible for the heat produced.

It should be noted that one cannot account for the very large accumulation of intermediary products noted above on the basis of fructose-1,6-diphosphate alone, since Macfarlane (9) has shown by analysis that the extent of the accumulation of this intermediate is relatively small and because much of the hexose diphosphate would not appear in the "intermediary fraction" in the present investigation since it is easily hydrolyzed to fructose-6-phosphate, a sugar derivative having a high reducing value.

Several experiments were carried out with yeast cells that had been starved by continuous aeration for 6 days. In the typical experiment shown in Fig 3, it can be seen that 21 per cent of the added glucose was assimilated, thus agreeing with the extent of assimilation in normal yeast cells. The only noteworthy difference from the normal yeast suspension is that the induction period of assimilation was shortened. This can be seen in Fig 4, which shows the assimilation curves of two normal and two starved yeast suspensions. The shortening of the induction period of assimilation

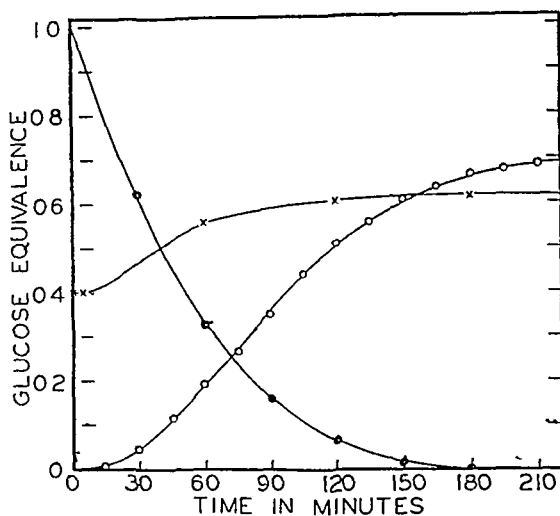


FIG 3 The anaerobic fermentation of 500 mg of anhydrous glucose by a yeast suspension starved for 6 days by continuous aeration. The same yeast suspension was used as in the experiment shown in Fig 1. ●, glucose concentration, ○, glucose fermented to carbon dioxide and ethanol, ×, total hydrolyzable polysaccharide content of the cells as glucose, the dotted line represents the intermediary products, estimated by difference.

was not brought about by differences in the rates of the over-all fermentations, since the glucose consumption was almost identical in the four experiments.

Van Niel and Anderson (4) assumed that the assimilated "products represent stored, non-reducing sugar polymers, such as glycogen, capable of being fermented at a considerably slower rate," in order to account for the nearly quantitative conversion of glucose to fermentation products upon extended anaerobiosis. This anaerobic utilization of reserve material by yeast cells is so slow that Winkler and Baumberger (3) found that little or no heat was produced. Spiegelman and Nozawa (10) conclude that intact yeast cells were unable to ferment their carbohydrate reserves, although the data showed a slow but definite carbon dioxide production. The results of an experiment in which 50 mg of glucose rather than the usual 500 mg were

fermented by a fresh yeast suspension are shown in Fig 5 There was a slow but definite carbon dioxide production both before the glucose had been

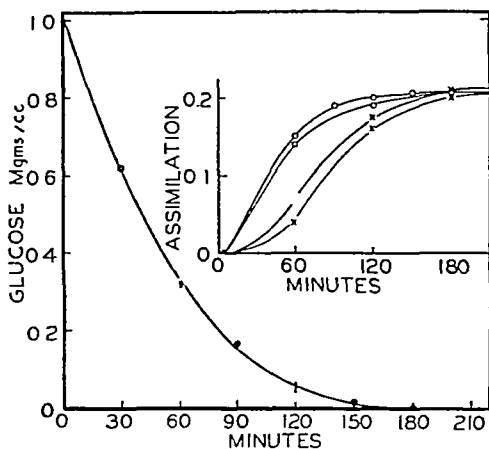


FIG 4 The assimilation of glucose during the anaerobic fermentation of 500 mg of glucose by starved and fresh yeast Data from the experiments shown in Figs 1 and 3 and from a duplicate experiment of each are presented The assimilation equals the increase in total hydrolyzable polysaccharide content X, fresh yeast, O, starved yeast, represents four points (two for the fresh and two for the starved suspensions) all falling within the bar

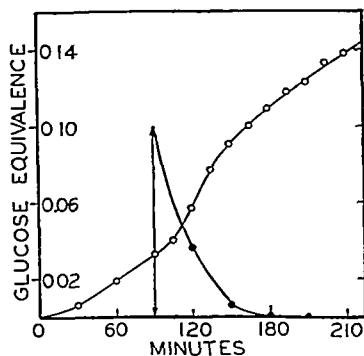


FIG 5 The anaerobic fermentation of 50 mg of glucose by a fresh yeast suspension The sugar was added at the arrow O, carbon dioxide, ●, glucose concentration

added and after the fermentation was complete In order to make this carbon dioxide production obvious, the scale used in Fig 5 is much larger than that in the preceding figures Upon inspection of the results shown in Fig 5, it can be seen that even more than the theoretical amount of carbon

dioxide may be produced if the concentration of reserve material is high in comparison to the concentration of the added glucose. This is in agreement with Guillemet (11) who found that more than the theoretical amount of carbon dioxide was produced by yeast rich in carbohydrate reserves.

#### DISCUSSION

From the experimental evidence presented, it would appear that in the over-all fermentation process there is a definite pattern of progressive changes in the rate of fermentation, in the rate of assimilation, and in the concentration of intermediary products, as if these were all integrated by the equilibria involved. Starvation modifies this pattern, as if the concentration of reserve material present influences the progress of fermentation.

#### SUMMARY

The anaerobic fermentation of glucose as carried out by living yeast cells was followed by a series of quantitative analyses of carbon dioxide, glucose, and stored carbohydrate during the course of the process. The following conclusions are drawn:

- 1 In the fermentation brought about by a commercial strain of bakers' yeast, 20 per cent of the added sugar is assimilated and 70 per cent is converted to carbon dioxide and ethanol.

- 2 Starved yeast cells synthesize the same proportion of the added sugar into cellular carbohydrate material as do cells which contain a normal amount of glycogen, but the synthesis is accomplished earlier in the process.

- 3 A surprisingly large quantity of intermediary products accumulates during the early phases of the fermentation. It was found by the difference between the disappearance of the glucose and the formation of the final products that as much as 40 per cent of the added sugar appeared as intermediary products at one stage of the fermentation studied.

- 4 The nearly quantitative yield of fermentation products obtained after long periods of anaerobiosis is due to the utilization of stored material after the complete disappearance of the substrate.

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# A CRYSTALLINE $\beta$ -AMYLASE FROM SWEET POTATOES\*

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In a previous publication (1) a method was described for preparing a crystalline amylolytic protein from sweet potatoes. This method has now been simplified and the yield of crystalline protein from most samples of tubers has been materially increased. Some data on the composition and properties of the crystalline product have been obtained and are also reported here.

Crystalline enzymes of carbohydrate hydrolysis have been reported twice in the literature, by Caldwell, Booher, and Sherman (2), and recently by Meyer, Fischer, and Bernfeld (3). Both preparations were of pancreatic amylase, and the former does not seem to have been consistently reproducible<sup>1</sup>. The purification and properties of  $\beta$ -amylase from grains have, however, been very extensively studied, for example, its separation from  $\alpha$ -amylase by Kneen, Sandstedt, and Hollenbeck (4), and its chemical behavior by Weill and Caldwell (5), who presented evidence of an indispensable sulfhydryl group. There is indeed such a large amount of literature on both  $\alpha$ - and  $\beta$ -amylases of grain that the properties of each enzyme (not pure but practically freed from each other) are remarkably well known.

Although grains have been generally regarded as the most practical source of plant  $\beta$ -amylase, Gore and Jozsa (6) have shown that amylase is of wide-spread occurrence in common plants, and that sweet potatoes are unusually rich therein. While repeating some of their survey of plants, we observed that the sweet potato amylase was almost entirely  $\beta$ -amylase, and that it was obtained quantitatively in the press juice. Further work showed among other things that the enzyme was rather stable, not destroyed by lead, and only slowly inactivated by acid. Furthermore, inactivation by acid was greatly delayed in the presence of ammonium sulfate. This stability in acid solutions appeared to offer an opportunity for

\* Enzyme Research Laboratory Contribution No. 111

<sup>1</sup> Attempts in our Laboratory to purify pancreas amylase led to preparations of 2 to 3 times the specific activity reported by Caldwell *et al.* (2) (on a protein basis), but crystallization was not successful. The enzyme was found as usual to be very unstable unless kept cold, and we missed the significance of the stabilizing factor used successfully by Meyer *et al.* (3).



extensive purification, and eventually it led to preparations that crystallized easily

*Method of Assay*—Since sweet potatoes were found to contain only traces of  $\alpha$ -amylase and small amounts of maltase which are lost early in the process of purification, only a method for the determination of  $\beta$ -amylase was required. The method used was Schwimmer's modification (7) of the determination of Kneen and Sandstedt (8). The unit of  $\beta$ -amylase employed here is that quantity of enzyme which liberates reducing substances equivalent to 1 milliequivalent of ferricyanide in 10 minutes at  $30^\circ$  in 30 cc of a solution of soluble starch at pH 4.8. An empirical curve (Fig. 1) shows

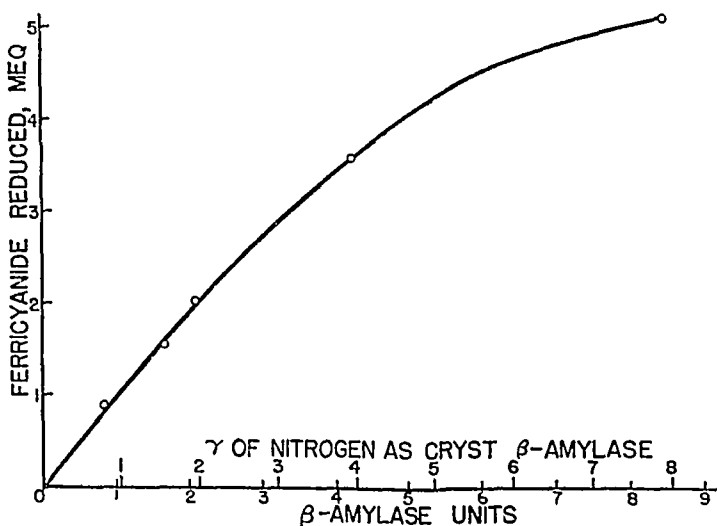


FIG. 1. Relation between  $\beta$ -amylase units and ferricyanide reduced in the method for assay. The curve was made with  $\beta$ -amylase crystallized five times. The quantities of crystalline enzyme corresponding to the scale of units are shown in micrograms of protein nitrogen.

the relation between enzyme units and milliequivalents of ferricyanide reduced.

The details of analysis describe the conditions which define the unit, and are as follows: 20 cc of a 2 per cent solution of Lintner starch, 0.02 M with respect to acetate, pH 4.8, are diluted to 30 cc with water and enzyme solution and incubated at  $30^\circ$  for 10 minutes. 3 cc of the digestion mixture are then added to 10 cc of 0.05 N ferricyanide in 0.2 M sodium carbonate solution, and the solution is placed in a boiling water bath for 20 minutes. The hot solution is then cooled in ice and acidified by the addition of 25 cc of an acid-salt mixture (70 gm of KCl, 20 gm of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , and 200 cc of glacial acetic acid in 1 liter). About 0.5 gm of KI is then added and the liberated iodine is titrated with 0.05 N thiosulfate as usual. The difference between a blank run and one with amylase is a measure of the reducing sub-

stances formed by the enzyme. When substances that themselves react with ferricyanide (for instance cysteine) are added to the enzyme to test their effect on it, a zero time control (in practice about 10 seconds later) is required. Otherwise, the ordinary blank was found sufficient.

*Preparation of Crude Concentrate*—Mature local sweet potatoes of the kind called Porto Rican red were found to be the richest in amylase. The concentration of enzyme increased rapidly as the tubers approached maturity and remained high thereafter, but after long storage the material was more difficult to handle. The potatoes were washed and coarsely ground. The pulp was squeezed dry in a hydraulic press, and the cake discarded because it contained practically no amylase. About 2 liters of juice were placed in an Erlenmeyer flask and heated and stirred in such a way that the temperature rose to 60–65° in 10 minutes. The flasks were then placed in cold running water. The temperature dropped to 40° in about 10 minutes and to 30° in half an hour. Some amylase was usually inactivated by this treatment. The heated juice was then stored for 4 days at 3–4° under toluene, during this time an appreciable increase in amylase activity always occurred. Small samples of the juice were next treated with basic lead acetate and filtered to determine the quantity of the reagent which precipitated between a quarter and a third of the enzyme present. The appropriate amount of suspension of basic lead acetate (200 gm per liter) was then stirred into the remaining juice, and the precipitate filtered off. Usually about 15 gm of lead acetate were required per liter of juice. Crystalline ammonium sulfate was added to the filtrate to 0.7 saturation, and the precipitated protein was collected and stored as a paste for further purification. Such pastes have kept satisfactorily for 3 years under refrigeration.

The method previously published by us (1) is satisfactory for the preparation of crystalline amylase from this paste. However, the yields depend much upon the condition of the particular sweet potatoes used, and perhaps upon the year of harvest. A slightly different method that gives a higher yield has been developed since by purifying the crude material more and omitting any extensive fractionation prior to crystallization. To this end, a concentration of the crude enzyme was made.

*Preparation of Purified Concentrate*—A convenient quantity of paste, usually 250 gm, was diluted with enough water to be 0.25 saturated with ammonium sulfate, brought to pH 5.0 to 5.1 by the addition of 2 N hydrochloric acid, and filtered through paper. The precipitate was dissolved in water, again made 0.25 saturated with ammonium sulfate at pH 5.0 to 5.1 (the total volume being about the same as before), and filtered again. The combined filtrates were found to contain most of the enzyme, and this was precipitated by the addition of solid ammonium sulfate to 0.7 saturation.

From 250 gm of crude paste about 60 to 65 gm of purified paste were usually obtained.

*Final Purification*—The purified paste was dialyzed in cellophane under toluene against running tap water for 2 or 3 days. The dialyzed solution was diluted so that 200 cc corresponded to 250 gm of the original (crude) paste, and then filtered with kieselguhr through a starch-free paper. The dilute was made 0.20 saturated by the addition of saturated ammonium sulfate solution, and the pH was adjusted to 5.0. If any precipitate formed, it was centrifuged out. The solution was then cooled to 8–10° and 2 N hydrochloric acid added to pH 3.25 to 3.30. It was then centrifuged at high speed for 6 to 8 minutes, and the (usually cloudy) supernatant at once brought to 0.25 saturation and pH 3.6 to 3.7 by adding saturated ammonium sulfate. At this point seed crystals were introduced. The solution was then stirred gently at room temperature, while saturated ammonium sulfate, pH 2.7 (as measured when diluted to 0.5 saturation), was added in small doses up to 0.33 saturation. Thereafter, stirring was continued for some hours (usually 12 to 18), when the concentration of sulfate was raised to 0.4 saturation.

When made in this way, the crystals are accompanied by varying but always considerable quantities of amorphous matter. They are easily separated from it owing to their remarkably high specific gravity. Crystals of  $\beta$  amylase settle in saturated ammonium sulfate solution.

*Recrystallization*—Most of the crystalline material was separated from the amorphous by centrifuging the 0.4 saturated suspension at a low speed for 8 to 10 minutes. Completeness of sedimentation was determined with the microscope. The crystalline sediment was washed twice in the centrifuge with 0.5 saturated ammonium sulfate solution (pH 3.7) and then suspended in water. When solution of the crystals was complete, more amorphous material and a little lead sulfate were centrifuged out. The solution was then made 0.20 saturated with ammonium sulfate (allowing for that already present with the crystals) and centrifuged again. The pH was adjusted, if necessary, to 3.6 to 3.7, the solution seeded, and ammonium sulfate added at room temperature with stirring. Saturated ammonium sulfate was added until 0.25 saturation, but thereafter a 0.66 saturated solution was added. Most of the crystal crop came out between 0.30 and 0.33 saturation and here the addition was made slowly. Sulfate was, however, added up to about 0.4 saturation.

The foregoing process was repeated for the third and subsequent crystallizations. Usually no amorphous material could be seen in the dark-field after the third crystallization. After five or six crystallizations, the crystals often became so large that a cover-glass broke them on the slide, and they were observed in a hanging drop.

TABLE I

*Yields and Specific Activity Obtained in Purification of Sweet Potato Amylase*

Material and solvent	Volume or weight	Activity units per g. of dry	Percent recovery	Specific activity in units per mg. protein <sup>a</sup>
45.5 kilos sweet potatoes ground and pressed, juice	22.5 liters	210	100	8
Heated to 60°	22.5 "	205	97	
Stored 4 days at 3°	22.5 "	227	108	
Filtrate from lead treatment	17.5 "	157	58	11
Precipitated at 0.7 saturation of ammonium sulfate, crude paste	1780 gm	1300	49	
250 gm. crude paste, 2 runs (a) and (b)				
Combine fractions soluble at 0.25 saturation	(a) 920 cc (b) 945 "	300 275	42 38	
Ppt. at 0.7 saturation, purified paste	(a) 67 gm (b) 64 "			
After dialysis and filtration	(a) 200 cc (b) 200 "	1260 1250	39 38	
" centrifuging at 0.20 saturation	(a) 250 " (b) 250 "	840	32	
Prior to 1st crystallization (0.25 saturation, pH 3.6)	(a) 256 " (b) 224 "	645 490	25 17	
1st generation crystals after dissolving (much amorphous material present)	(a) 30 " (b) 25 "	3120 3280	14 13	830 950
Prior to 2nd crystallization	(a) 38 " (b) 40 "	2640 2240	15 14	
2nd generation crystals after dissolving (little amorphous material)	(a) 40 " (b) 30 "	1880 2760	12 13	990 1030
Prior to 3rd crystallization	(a) 50 " (b) 40 "	1590	12	
3rd generation crystals stored in 0.66 saturated ammonium sulfate, pH 3.7 (trace only of amorphous material)	(a) 10 " (b) 10 "	6540 6250	10† 10	1130 1100

<sup>a</sup> This value is really a ratio and not claimed to be more than a good approximation.  
† Equivalent to 283 mg. of protein.

The scheme of purification and crystallization is outlined in Table I for the purpose of giving the yields and specific activities obtained.

Rapidly grown crystals (Fig. 2) are usually twelve-sided figures, apparently perfect, but when they are grown slowly in the cold from a con-

centrated solution of protein, they often appear as hexagonal prisms capped by pyramids of the same order. Both crystal forms appear to be uniaxially refractive. When a suspension of crystals in ammonium sulfate (pH 3.7) was evaporated to dryness at room temperature, and the residue resuspended in ammonium sulfate solution, it was found that many of the crystals



Fig. 2 Crystals of sweet potato amylase ( $\times 400$ )

crystals were uninjured. (One dried preparation still retained a trace of its original activity and showed a corresponding decrease in activity per mg of protein N.)

*Tests for  $\alpha$ -Amylase, Maltase, and Phosphatase*—Sweet potato juice contains traces of  $\alpha$ -amylase and maltase and a large quantity of inorganic phosphatase.  $\beta$ -Amylase crystallized five times was tested for these enzymes.

No  $\alpha$ -amylase was found by the sensitive method of Schramm (1), with 10 mg of enzyme protein and a digestion time of 4 hours.

amount of enzyme used (about 200 units) was enough to have produced about 10 times the limit dextrin used in the test in 10 minutes.

Maltose was tested for by permitting 10 units of crystalline enzyme to act on 50 mg of pure maltose in 10 cc of 0.05 M acetate buffer (pH 5.2) for 30 minutes at 40°. Thereafter, the glucose formed was estimated by the procedure of Tauber and Kleiner (10), however, a photoelectric colorimeter was used. The glucose found was 0.65 mg. This value is too small to be regarded with much confidence, for the detection of such small amounts of glucose in the presence of much other reducing sugar is not

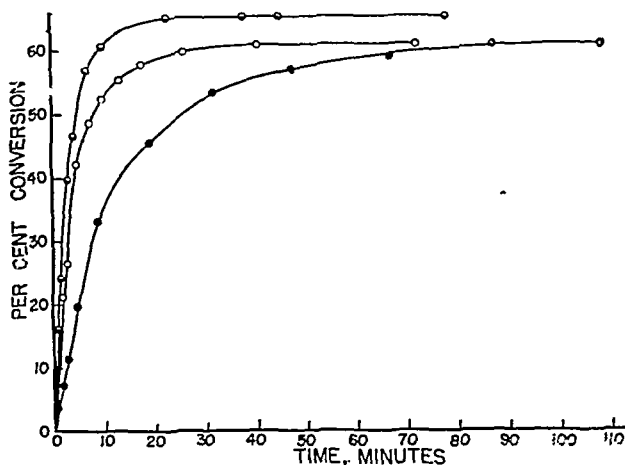


Fig. 3 Course of starch conversion (O) by purified wheat  $\beta$ -amylase, (□) or de  $\beta$  amylase (sweet potato juice), (●) sweet potato amylase crystallized five times

very satisfactory. If the crystals show maltase activity at all, it must be very small.

Further evidence of the absence of maltase in the crystalline material is furnished in Fig. 3 by a comparison of the extent of starch conversion produced by three enzyme preparations diluted to contain approximately the same  $\beta$ -amylase activity. These preparations were (a) sweet potato juice dried in a vacuum while frozen and redissolved in water for use, (b) crystalline  $\beta$ -amylase, and (c) a commercial preparation of  $\beta$ -amylase prepared from wheat by the procedure of Sandstedt, Kneen, and Bligh (11) and demonstrated to be free from  $\alpha$ -amylase. This is the preparation customarily used in the assay of cereals for  $\alpha$ -amylase. The conversion was determined by the method used here for assay. An empirical curve (not shown) was made with pure maltose and used to convert the ferricyanide reduced by the corresponding quantities of maltose.

The original sweet potato juice gave ultimately a higher reducing value than either the crystalline or the wheat preparations. This is presumably

TABLE II  
*Analysis of Crystalline  $\beta$ -amylase*

Component	Per cent	Notes
Arginine	6.0	Hydrolyzed 20 hrs. with constant boiling HCl, thereafter by Sakaguchi method as modified by Thomas, Ingalls, and Luck (14), extrapolation after Brand and Kassell (15)
Tyrosine	7.0	Hydrolyzed 20 hrs. with constant boiling HCl, thereafter by modified Millon (Block and Bolling (16))
Cystine + cysteine (as cystine)	0.79	Hydrolyzed 6 hrs. with constant boiling HCl, determination after Sullivan, Hess, and Howard (17)
Methionine	4.32	Hydrolyzed 20 hrs. with constant boiling HCl, method of McCarthy and Sullivan (18), modified*
Amino N	0.83	Van Slyke, 3 min
Amide "	1.16	Hydrolyzed 5 hrs. in 2 N HCl
Protein N	14.8	Trichloroacetic acid precipitation, Kjeldahl
Total N†	15.1	Kjeldahl, after dialysis of 7 times crystallized enzyme, sample dried at 105° in air to constant weight
" "	15.2	Dialysis, Dumas, sample dried at 105° in air to constant weight
Ash	0.66	

\* Dr. Bernard Axelrod, of this Laboratory, has found that when the addition of the strong acid to the reaction mixture is made by the procedure of McCarthy and Sullivan considerable variation in the final color may be encountered. This difficulty, especially serious with the blanks and with moderate concentrations of methionine, was obviated by precooling the two solutions in separate tubes in an ice bath, mixing them quickly by pouring back and forth, and then proceeding by the method of reference. In the determinations shown here, the blank was the same for all five aliquots of the hydrolyzed protein. With the smallest sample it amounted to about 40 per cent of the total reading and correspondingly less with larger samples.

† The nitrogen determination (17.48 per cent), reported by us previously (1), cannot be repeated and is evidently an error.

because of the maltase it contains. On the other hand, there is a remarkably close correspondence between the end-points of the other two preparations. The observed conversion (60 per cent) is close to the value generally

accepted for the action of  $\beta$ -amylase in cereals on this type of starch.<sup>2</sup> No evidence for or against the identity of the residual dextrans formed by the two enzymes has yet been obtained.

Phosphatase was measured by the *p*-nitrophenyl phosphate method of Axelrod (13) with 50  $\beta$ -amylase units (0.044 mg of protein nitrogen). This quantity was found to contain less than 0.25 phosphatase unit, which is about the lower limit of sensitivity for the method. The ratio of phosphatase units to amylase units was therefore about 0.05, whereas the ratio in the original sweet potato juice was slightly over 8.0. Despite the purification of the protein before crystallization, the mother liquor of the first crystallization was rich in phosphatase, and the thrice crystallized protein still contained small but measurable amounts.

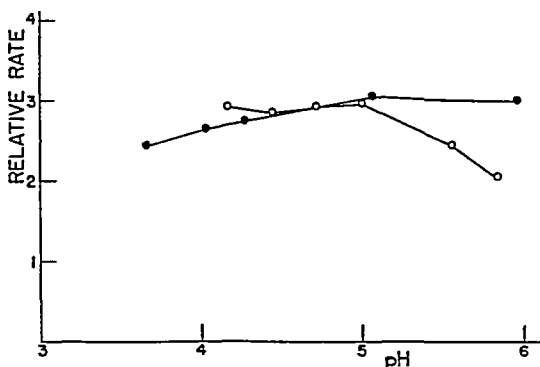


FIG. 4. pH optimum of sweet potato amylase crystallized three times, O acetate buffer, ● citrate buffer.

*Analytical*—The results obtained in the determinations of several constituents of a thrice crystallized preparation (specific activity about 1100) are given in Table II. The protein contains more than an average amount of tyrosine and very little cystine and cysteine.

The absorption spectrum of the solution of material crystallized three and six times gave no indication of a known prosthetic group. The solutions were slightly yellowish, although the color was decreased by successive crystallizations. The usual protein absorption, presumably due here to

<sup>2</sup> Over a wide range the extent of conversion was practically independent of the quantity of crystalline sweet potato amylase used. Thus in 60 minutes at 30°, with 30 cc. of the starch substrate previously described, 3 units of the same enzyme preparation used above gave 59.0 per cent conversion, 30 units gave 60.3 per cent, and 300 units gave 60.8 per cent. The method used for the determination of reducing sugar in this experiment was not that given in the text, but a modification of the Willstätter-Schudel method described by Jansen and MacDonnell (12).



tyrosine, was noticed. The spectrum of the ash was, except for a trace of lead, practically that of the solids in the tap water used for dialysis.

There is thus no indication that the enzyme contains a light-absorbing prosthetic group or is a heavy metal complex.

*Nitroprusside Reaction*—The usual nitroprusside test on about 3 mg portions of protein was practically negative both before and after reduction with cyanide. In the presence of guanidine, however, it was strongly positive but not visibly increased by cyanide. Protein coagulated by heat also gave a strongly positive nitroprusside test without guanidine. It may be concluded that all the S—S and —SH sulfur is masked, and that a large proportion of it exists as —SH in the native protein.

*pH Optimum*—The activity of enzyme crystallized three times at various pH levels was determined with citrate and with acetate buffers, each 0.10 M in the reacting system. The results are given in Fig. 4. Between pH 4 and pH 5 the activity is not markedly affected by changes in the pH. Beyond this range, however, it is obviously affected by the character of the anions present. Ballou and Luck (19) previously found a similar picture in their very careful work on the pH optimum of wheat  $\beta$ -amylase.

#### SUMMARY

A crystalline protein exhibiting a high activity as  $\beta$ -amylase has been prepared from sweet potatoes. The yield is substantial, about a tenth of the original activity remains after three crystallizations. Through most of its purification the enzyme was accompanied by a phosphatase and by some carbohydrate, but neither is present after several crystallizations.

The enzyme behavior of the crystalline protein corresponds closely to what is generally regarded as  $\beta$ -amylase. The pH optimum is very broad, Lintner starch is broken down to an extent of about 60 per cent, and no  $\alpha$  amylolytic activity has been detected.

Some observations on the composition of the crystalline enzyme are also reported. Spectroscopic examination has given no indications that the enzyme contains an essential heavy metal or a recognized prosthetic group.

The authors desire to thank Dr. E. J. Eastmond and Mr. G. F. Bailey of the Western Regional Research Laboratory for the spectroscopic examinations, Dr. Bernard Axelrod of this Laboratory for the phosphatase determinations, and Dr. Sigmund Schwimmer of this Laboratory for the tests for  $\alpha$ -amylase, maltase, and the extent of starch conversion.

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2

3

# THE COMBINATION OF ADENINE, ADENOSINE, AND ADENYLIC ACID WITH SERUM ALBUMIN

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The importance of adenine derivatives in many biological systems makes it desirable to have information on the interactions of some of these compounds with proteins. It has been demonstrated that adenosine and adenylic acid may be removed from solution by heat-coagulated proteins and that adenylic acid may be bound by a protein in the sol state at a pH acid to the isoelectric point (1). Evidence of binding at a pH above the isoelectric point, at which both protein and adenylic acid would be anionic, has been lacking. It has seemed desirable, therefore, to make quantitative investigations of such interactions, similar to those carried out previously with other organic molecules (2).

## EXPERIMENTAL

*Materials*—Bovine serum albumin was Armour's crystallized product. Its water content was determined by heating in an oven at 110°.

Adenine hydrochloride, adenosine, and adenylic acid (adenosine-3-phosphate) were obtained from the Schwarz Laboratories. At pH 7.6, all showed absorption peaks at 2600 Å and molecular extinction coefficients of 12,600, 14,600, and 14,600, respectively, in substantial agreement with the values reported by Mitchell and McElroy (3). The adenylic acid was also titrated with base and showed a  $pK_2$  of 6.23 as well as an equivalent weight corresponding to  $100 \pm 0.5$  per cent purity. Nitrogen analysis, however, gave a slightly low value, 19.5 per cent being found as compared to 20.17 per cent calculated from the formula of adenylic acid.

Buffers of pH 7.6 were prepared from Baker's C.P. Analyzed grade of phosphate salts and were approximately 0.1 M.

*Methods*—Cellophane bags were prepared from commercial sausage casing and were filled with a measured amount of a protein solution, usually near 8 per cent concentration. The bag was immersed in a solution of the purine derivative and placed in a refrigerator at approximately 5° for a period of 72 hours, an interval sufficient for the attainment of equilibrium. The bag was then removed and the external solution analyzed for the purine compound by means of the optical absorption at 2600 Å. For each concentration of purine, a control tube was prepared also, which differed from the primary tube only in that the former contained buffer rather than a protein

solution inside the bag. By this method it is possible to minimize any errors arising from binding of the purine by the cellophane membrane.

Optical densities were measured in the Beckman ultraviolet spectrophotometer with 1 cm cells.

## RESULTS AND DISCUSSION

The method of calculation of the moles of bound purine per mole of total protein from the experimental data has been described previously (2). One additional consideration is necessary in these measurements. Since the concentration of protein is quite high, a significant Donnan effect will be present in the equilibrium with the adenylyate ion. If a correction is not made for this effect, the binding calculations will give low values. The magnitude of the Donnan correction was estimated, therefore, from data in the literature (4-7), and the ratio of (divalent) anion concentrations outside and inside the bag, respectively, was taken as 1.08. No correction was made for the adenine or adenosine, since their hydrochlorides have pK values of 4.1 and 3.6 (8), respectively, and hence they would exist as neutral molecules in a solution of pH 7.6.

The extent of binding of these adenine derivatives as a function of the concentration of the free molecule has been summarized in Fig. 1. In each case the average number of bound molecules on each protein molecule increases with increasing concentration of the free purine. It is also immediately evident from Fig. 1 that the addition of the phosphate group to the adenosine increases the binding in a much more pronounced fashion than does the addition of the ribose residue to the adenine.

Several experiments were tried also to determine the extent of binding of adenylic acid by bovine  $\gamma$ -globulin. No significant binding was observed. Such behavior parallels that observed previously with other organic anions (9).

The quantitative data on binding by albumin may be correlated in terms of the laws of equilibrium by the following procedure. Since the average number of bound molecules per molecule of protein is so very much less than 1, it is not necessary to write general equations for multiple binding, but instead it is sufficient to describe the equilibria by a single equation of the form



where  $P$  represents the protein and  $A$  the adenine derivative. If the moles of bound organic molecule per mole of total protein are represented by  $\nu$ , then it is clear from the definition that

$$\frac{(PA)}{(P) + (PA)} \quad (2)$$

Simple algebraic manipulation leads to the following expression for the equilibrium constant for reaction (1)

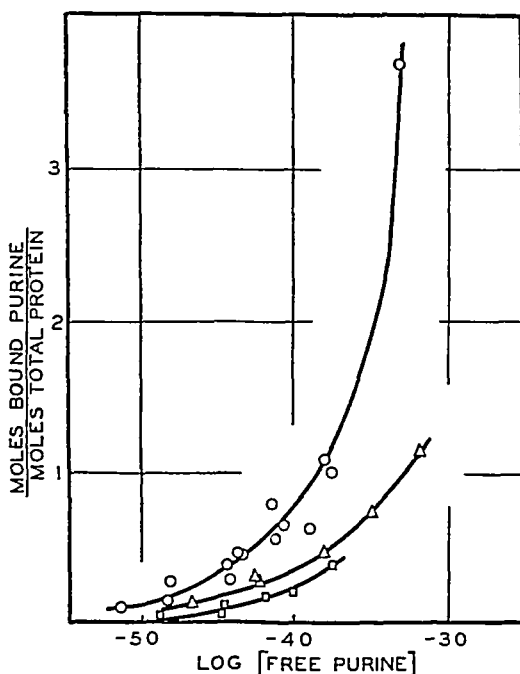


FIG 1 Binding of some purine compounds by bovine serum albumin at 5° and pH 7.6 □ adenine, △ adenosine, ○ adenylic acid

TABLE I

*Free Energies of Binding of Some Adenine Derivatives by Bovine Serum Albumin at 5° and pH 7.6*

Substance	$\Delta F^\circ$
	<i>calories per mole</i>
Adenine	-3080
Adenosine	-3370
Adenylic acid	-3810

$$l_1 = \frac{r}{1-r} \frac{1}{(A)} \quad (3)$$

From the equilibrium constants, the free energies of binding may be evaluated through the thermodynamic expression

$$\Delta F^\circ = -RT \ln k \quad (4)$$

The free energies of binding have been calculated and are assembled in Table I. The values are far lower than those observed with some of the azo dyes (2). The more polar nature of the purine compounds apparently tends to keep them in the water. On the other hand, it is of interest to note that the phosphate group, notwithstanding its negative charge, increases the binding energy to a greater extent than does the addition of a ribose residue, despite the much larger size (and hence increased van der Waals interaction) of the latter. However, such behavior is in agreement with the point of view that part of the stabilization energy of the anion-protein complex is derived from electrostatic interaction between the negatively charged organic ion and a positively charged quaternary nitrogen on the protein molecule (9).

#### SUMMARY

Quantitative data have been obtained on the binding of adenine, adenosine, and adenylic acid, respectively, by bovine serum albumin. Free energies of binding have been calculated and are  $-3080$ ,  $-3370$ , and  $-3810$  calories per mole, respectively.

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# THE EFFECT OF TEMPERATURE ON THE pH OF BLOOD AND PLASMA IN VITRO\*

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Although the electrometric technique of measuring blood pH has been simplified and rendered quite accurate, there still appears to be some doubt about the numerical value of a correction to be applied to the observed pH in cases in which the temperature of the blood sample is not 38° (1-3). The acidity of blood *in vivo* varies to such an extent with temperature that for accurate determination of its pH the sample should be measured at body temperature. Due to the inconvenience, however, of working with temperature-controlled apparatus it has been the practice to take measurements at room temperature and then make the appropriate corrections. In many clinical laboratories plasma or serum is used instead of whole blood, which can introduce certain complications in the interpretation of results. Unless we have precise knowledge of the effect of temperature on the pH of whole blood and plasma, it is difficult to compare measurements taken under these varying circumstances. A systematic study was therefore undertaken on a large number of samples under controlled conditions with glass electrodes capable of giving results dependable to 0.01 of a pH unit.

## EXPERIMENTAL

Blood was drawn anaerobically from the heart or femoral artery in animals and from the antecubital vein in human subjects, coagulation being avoided either by defibrination under oil or by addition of potassium oxalate solution to the syringe. Coagulation in itself does not cause a change of blood pH (4), but difficulty in cleaning made it necessary to avoid clot formation within the capillary tubing of the apparatus.

Concentrations of 0.1 to 0.2 per cent oxalate are sufficient for the purpose and do not alter pH or disturb ionic relationships between cells and plasma (5). A blood sample so treated and kept in the syringe will maintain a constant pH for about 30 minutes, but if it is necessary to delay the pH measurement for 24 to 48 hours it is advisable to add 0.06 per cent NaF as well as oxalate and to chill the sample immediately. This procedure

\* Data taken from a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Yale University, 1941.

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inhibits the glycolytic reactions which otherwise produce a drop in pH amounting to a large part of a unit in the course of a few hours

The basic data were obtained by dividing the sample into two portions and making the pH measurements simultaneously at 38° and at room temperature. With the use of the temperature-controlled vessels and semi-micro glass electrodes described by Nims (6), samples of 1 ml. were found ample for duplicate pairs of determinations. As shown in Fig. 1, the E.M.F. of each glass electrode in its blood sample is measured potentiometrically with respect to the saturated calomel half-cell, balance being achieved with the aid of an amplifier (7) which permits the potentiometer to be read to 0.2 millivolt. The voltage reading given by each blood sample is con-

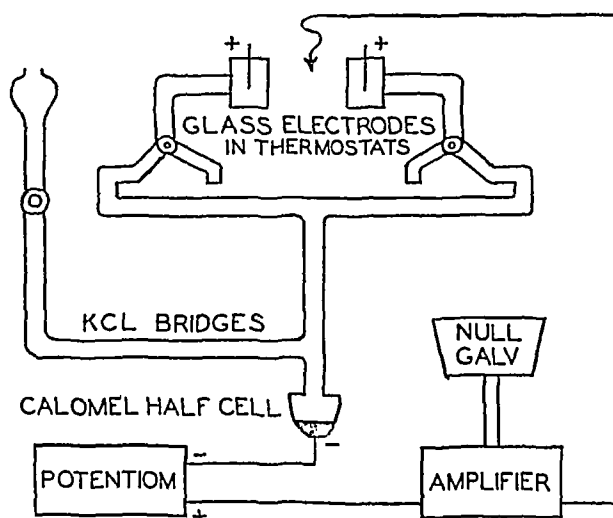


Fig. 1 Potentiometric determination of pH, blood samples maintained at body and room temperatures. Liquid junctions are formed within the stop-cocks.

verted to pH units by interpolation between the readings given by two buffers of known pH (8).

The first problem was the general nature of the relationship between pH and temperature in any given blood sample. It was found that pH rose linearly with a fall in temperature over the range studied from 38–18°. In Fig. 2 are presented the results on a few samples taken at random. These measurements were made on each sample with three glass electrodes, at three temperatures simultaneously. Since the lines for whole blood are practically parallel, we conclude that the rate of change of pH with temperature is independent of the initial value of pH at 38°. A more extensive investigation later showed that the slope of the line does not change appreciably when the initial pH at 38° varies from 7.25 to 7.45, which are about the limits of physiological variation.

Next, the question of reversibility was studied. It was found that when fluoride was employed to prevent glycolysis the blood sample could be repeatedly warmed and cooled from 38–3° without altering the pH at 38° more than 0.01 unit.

Table I summarizes the principal findings on whole blood. Since the relation between blood pH and temperature is linear, the "temperature coefficient" (pH units per degree centigrade) was calculated by dividing the difference in pH (at body and room temperatures) by the difference in temperatures. In each of these series no attempt was made to establish a "normal" value of blood pH at 38° or at any other temperature. In fact the original pH was often deliberately altered to some extent so that the

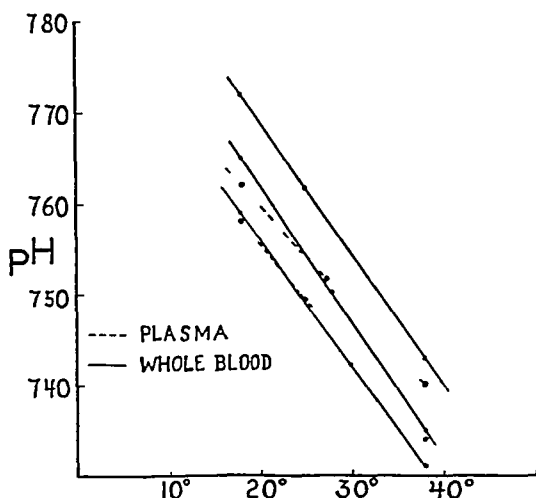


FIG 2 Examples of linear relationship between pH and temperature for whole blood and for plasma

mean value of the temperature coefficient would be representative of a series of normal and abnormal blood specimens.

In this connection a study was made of the effect of changes in the concentrations of the principal buffer systems of the blood. The hemoglobin, plasma protein, and bicarbonate concentrations of different samples were raised or lowered about 50 per cent by addition of erythrocytes or by dilutions with plasma or saline. In each case no significant change occurred in the slope of the line. Hence we may assume that the numerical value of the temperature coefficient for pH, as given below, applies both in health and disease.

Since the means for blood of various species are so close to each other,

the data were subjected to an analysis of variance, which showed that the variance of measurements within a species was greater than the variance of means among the species (by a factor of 2.3). Hence we are justified in proposing a single mean value of  $-0.0147$  pH unit per degree centigrade for whole blood of man, dog, cat, and rabbit. Or in general where  $t$  is the temperature at which blood pH is read

$$\text{Blood pH}_{38} = \text{pH}_t - 0.0147(38 - t) \quad (1)$$

As might be anticipated from the absence of a protein concentration effect, no significant difference could be found between the behaviors of serum and plasma. Both exhibit the characteristic linear relationship

TABLE I  
*Whole Blood of Various Species*

Source	No. of samples	Mean temperature coefficient*	Standard deviation
T B R	34	0.0149 <sub>0</sub>	0.00055
Miscellaneous, human	18	0.0146 <sub>5</sub>	0.00060
Dogs	43	0.0146 <sub>0</sub>	0.00065
Cats	10	0.0149 <sub>0</sub>	0.00065
Rabbits	8	0.0146 <sub>5</sub>	0.00055
Weighted mean		0.0147 <sub>0</sub>	0.00061

\* Over the range 38° to room temperature (18–24°)

TABLE II  
*Plasma or Serum of Various Species*

Source	No. of samples	Mean temperature coefficient	Standard deviation
T B R	15	0.0115 <sub>0</sub>	0.00025
Miscellaneous, human	15	0.0120 <sub>0</sub>	0.00050
Dogs	23	0.0107 <sub>5</sub>	0.00110
Rabbits	7	0.0138 <sub>0</sub>	0.00025

(Fig. 2) between pH and temperature, independent of initial pH at 38°. As Table II shows, however, the numerical value of the temperature coefficient is lower than that of whole blood and there is a certain amount of species variability. We confirm an observation of Yoshimura (3) that the temperature coefficients of rabbit blood and plasma are almost the same. For human plasma a formula similar to that of blood may be written

$$\text{Plasma pH}_{38} = \text{pH}_t - 0.0118(38 - t) \quad (2)$$

*Relation between Whole Blood and Plasma*—Normal physiological or "true" plasma is here defined as that plasma which is in physicochemical

equilibrium with the erythrocytes at  $38^{\circ}$ . Its pH is identical with that of whole blood at  $38^{\circ}$ , since the erythrocytes behave merely like inert suspended bodies with respect to the electrodes. "Derived" plasma is defined as plasma obtained from whole blood by separation at any temperature other than  $38^{\circ}$ . It differs in pH and in chemical composition from true plasma. Moreover a derived plasma warmed to  $38^{\circ}$  will not yield the same pH as the true plasma, because of the difference in temperature coefficients between whole blood and plasma. The relationships are graphically illustrated in Fig 3.

The solid line for whole blood is drawn with the slope of  $-0.0147$ , with  $\text{pH}_{38}$  set equal to 7.40. The broken line for true plasma is drawn with the

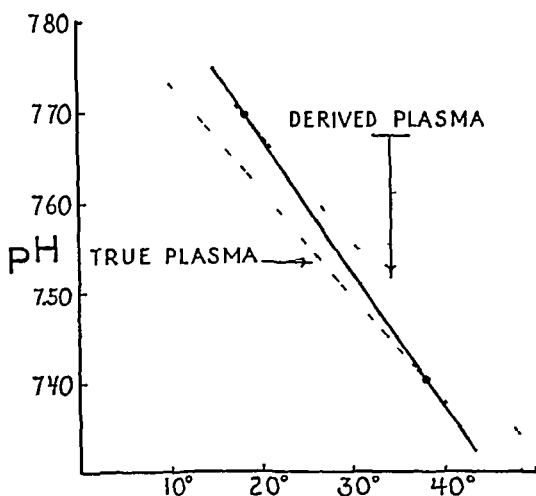


FIG 3 The influence of the temperature of centrifugation, showing how two plasma samples separated from the same whole blood can have different pH values

slope of  $-0.0118$  and must intersect the solid line at  $38^{\circ}$ . Another broken line with the same slope is drawn to represent a derived plasma obtained at  $18^{\circ}$ . In this particular case at a given temperature the difference in pH between true and derived plasma is almost 0.06. If the temperature of separation were  $3^{\circ}$ , this difference would amount to about 0.10.

To avoid errors in calculating blood  $\text{pH}_{38}$  from measurements on derived plasma formulae (1) and (2) are combined to give a more general relation-

$$\text{Blood } \text{pH}_{38} = \text{plasma pH} - 0.0118(38 - t_m) - 0.0029(38 - t_c) \quad (3)$$

where  $t_c$  is the temperature at which the blood was centrifuged, and  $t_m$  is the temperature of the plasma when its pH is measured.

It may be well at this point to call attention to a misconception on the part of some who use commercial pH meters that are equipped with "temperature correction" controls. Such controls are designed to compensate for temperature variation in the factor  $RT/F$ , which appears in the well known formula relating EMF and hydrogen ion concentration, so that the meter scale can be read directly at all ambient temperatures. Simply setting the pointer at 38° does not solve the problem of finding  $\text{pH}_{38}$  while the sample is at room temperature.

One might expect to find differences in the plasma concentrations of other electrolytes as a function of the temperature of separation. A short series of preliminary experiments was therefore made to compare the chloride and  $\text{CO}_2$  contents of a true plasma with those of a derived plasma. For chloride the loss amounted to about 3 per cent, but for  $\text{CO}_2$  it was quite variable (2.5 to 14.5 per cent), probably because the samples were not uniform with respect to pH and  $\text{CO}_2$  content. There is enough evidence, however, to indicate the necessity of a standard temperature of centrifugation if results of plasma analyses are to be comparable.

The author wishes to express his appreciation for the unfailing advice and assistance given by Dr L. F. Nims, Dr D. I. Hitchcock, and Dr P. H. Lavietes. The figures are the work of Mr A. Freeman.

#### SUMMARY

The temperature coefficients of pH for whole blood of man and laboratory animals are found to be the same, but minor species differences exist in their plasmas. The numerical value of the temperature coefficient is constant between 38–18° and independent of abnormal concentrations of hemoglobin, plasma protein, and bicarbonate.

A formula is presented by which the original pH of a blood sample can be calculated from a measurement of its plasma pH at room temperature. Relations between whole blood, true plasma, and derived plasma are discussed.

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# INACTIVATION OF ARGINASE BY PROTEIN DENATURANTS

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Anson and Mirsky (1) showed that, when hemoglobin is denatured by neutral sodium salicylate, the relation between salicylate concentration and percentage denaturation (the latter measured by the change in green light absorption) is represented by an S-shaped curve. It might be anticipated that with other proteins and other comparable denaturants a similar relation would prevail. If the protein were an enzyme, denaturation would involve loss of activity, and the curve of percentage or fractional inactivation *versus* concentration of denaturant might be expected to be again a sigmoid. In the case of the enzyme arginase this expectation, we have found, is realized not only in the action of sodium salicylate, but also in that of at least three other denaturants: potassium thiocyanate, guanidine hydrochloride, and sodium dodecyl sulfate (Duponol C). With the last of these it seems likely that loss of activity and extent of denaturation are exactly commensurate. With the others the situation is perhaps not so simple.

## Method

In studying the effect upon arginase activity of the four substances mentioned we adopted the procedure which we had already applied to the measurement of the inhibitory effect of amino acids (2).

Into each of a series of urea tubes were measured 2 ml. of 1.875 per cent arginine hydrochloride in phosphate-phenolsulfonate buffer solution (0.5 M, pH 8.4). To the first tube there were added 5 ml. of water, to each of the others 5 ml. of a solution of the denaturant under test, the concentration of the added solution increasing stepwise from tube to tube up to an appropriate maximum. With potassium thiocyanate this maximum was 41.6 per cent, with sodium salicylate 30 per cent, with guanidine hydrochloride 24.45 per cent, and with Duponol C 0.2 per cent. The mixtures having been brought to 37° in a water thermostat were treated each with 1 ml. of a cobalt-activated arginase solution of convenient strength. After an incubation period of, in each case, 30 minutes, the reaction was stopped, the quantities of urea produced were determined, and the results were translated into terms of arginase activity by reference to our standard curve for an arginine concentration of 0.0223 M (3). This procedure is justifiable

only if the form of the curve relating enzyme quantity and action within 30 minutes is unaffected by the presence of the inactivating agent. We have satisfied ourselves that this is true for salicylate, and have assumed it to be so for the other agents also.

The urea determinations were made by the urease method. Since that enzyme itself is inactivated in greater or less degree by each of the denaturants studied, care was taken to employ in every series of experiments

TABLE I  
*Inactivation of Arginase by Sodium Salicylate*

Each mixture contained 5 ml. of a sodium salicylate solution of the concentration indicated in the first column, 2 ml. of a buffer solution containing 1.875 per cent of arginine hydrochloride, and 1 ml. of arginase solution. Total volume 8 ml., pH 8.4, arginine concentration 0.0223 M.

Concentration of sodium salicylate		Urea N found	Arginase corresponding	Fractional inactivation, $1 - \alpha$
As added	Final			
per cent	M	mg	units	
0	0.000	2.852	12.66	
2	0.078	2.852	12.66	0.000
4	0.156	2.818	12.38	0.022
6	0.235	2.764	11.92	0.058
8	0.313	2.678	11.22	0.114
10	0.390	2.530	10.23	0.192
0	0.000	2.612	10.79	
12	0.469	2.197	8.03	0.256
14	0.547	2.025	7.05	0.347
16	0.625	1.734	5.58	0.483
18	0.703	1.407	4.18	0.612
0	0.000	2.894	13.01	
20	0.781	1.242	3.53	0.718
22	0.859	0.938	2.48	0.809
24	0.937	0.604	1.49	0.885
26	1.015	0.360	0.82	0.937
28	1.093	0.181	0.40	0.969
30	1.171	0.119	0.25	0.981

a demonstrably adequate excess. The determination was usually completed by aeration of ammonia and titration. This procedure could not be used in the presence of guanidine, for that substance itself yields ammonia upon treatment with alkaline carbonates. In the guanidine series, accordingly, we employed, as the final step, the manometric determination of  $\text{CO}_2$  (4), making the reaction mixture up to 15 ml., and applying the urease to a 5 ml. aliquot.

## Results

The results obtained with *sodium salicylate* are recorded in detail in Table I. They are derived, it will be observed, from three separate experiments, each covering one part only of the whole range of salicylate concentrations. The quantity of arginase employed was not identical in all experiments, but the data are reduced to a uniform basis in the last column of Table I, where, under the designation "Fractional inactivation" and the symbol  $1 - \alpha$ , the diminution of arginase activity resulting from each given concentration of salicylate is expressed as a fraction of the activity in the absence of the denaturant. Although the first and last of the three experiments were separated, as it happened, by an interval of 22

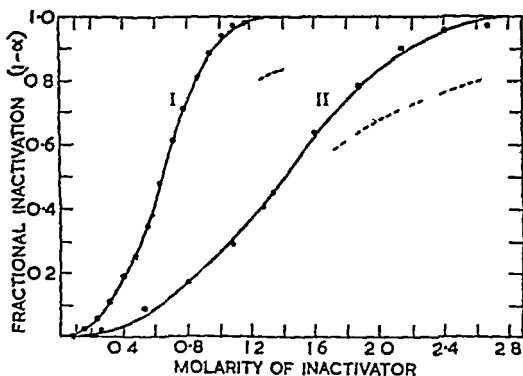


FIG 1 Inactivation of arginase by sodium salicylate (Curve I) and potassium thiocyanate (Curve II). The early part of Curve I is drawn to fit the equation  $1 - \alpha = [\text{NaC}_7\text{H}_5\text{O}_2]^2 / ([\text{NaC}_7\text{H}_5\text{O}_2]^2 + 0.427)$ , the early part of Curve II to fit  $1 - \alpha = [\text{KCNS}]^2 / ([\text{KCNS}]^2 + 2.72)$ . In each case the curve representing the given equation is completed as a broken line.

days, the combined results form a completely concordant series. This is made evident in the graph of Fig 1, where fractional inactivation plotted against molar concentration of salicylate is seen to yield a smoothly continuous curve.

Fig 1 exhibits also the outcome, similarly interpreted, of a single inclusive experiment with *potassium thiocyanate*, while Figs 2 and 3 represent results obtained with *guanidine hydrochloride* and *Duponol C* respectively. On the guanidine graph each of the first six points is an average derived from three separate experiments, one only of which was extended to include concentrations higher than 1.0 M. For Duponol C the points are drawn from two experiments, one covering concentrations (calculated as for pure Na dodecyl sulfate) from 0.000103 to 0.00103 M, the other the



overlapping range 0.000517 to 0.00414 M. It may be added that in all cases additional experiments gave results sufficiently consistent with those here exhibited.

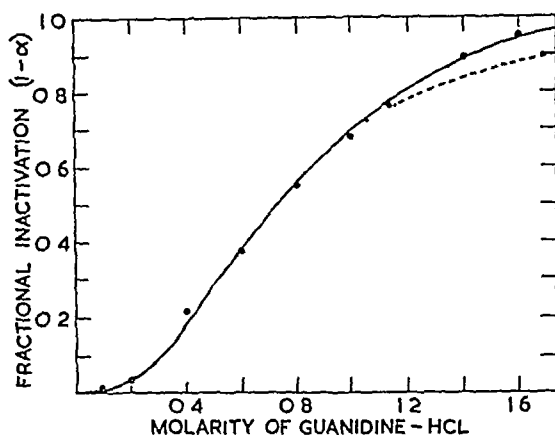


FIG. 2. Inactivation of arginase by guanidine hydrochloride. Up to a fractional inactivation of 0.7 the line drawn through the experimental points conforms to the equation  $1 - \alpha = [\text{CH}_5\text{N}_3\text{HCl}]^2 / ([\text{CH}_5\text{N}_3\text{HCl}]^2 + 0.451)$ . The later course of the curve representing this equation is shown as a broken line.

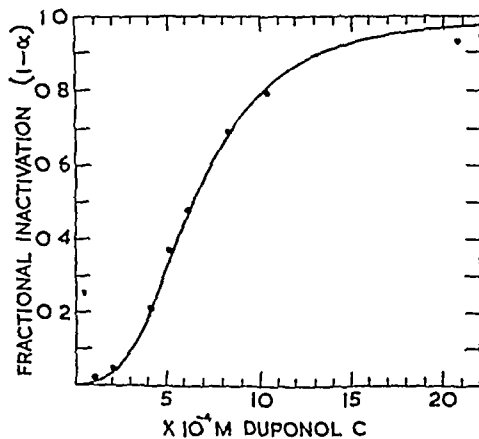


FIG. 3. Inactivation of arginase by Duponol C. The line drawn represents the equation  $1 - \alpha = [\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}]^2 / ([\text{C}_{12}\text{H}_{25}\text{O}_4\text{SNa}]^2 + 2.63 \times 10^{-10})$ .

#### DISCUSSION

The results shown, although reproducible, were obtained by a method to which there is one fairly obvious objection. Denaturation by substances like sodium salicylate is neither an instantaneous nor an uncomplicated reaction. According to Anson and Mirsky (1) it involves a reversible change, reaching completion in about 20 minutes at 25°, followed (per-

haps accompanied) by a slower irreversible one. Ideally, therefore, activities should have been measured only after an interval long enough to permit the establishment of an equilibrium between native and denatured arginase, yet so short that irreversible changes had made only negligible progress. Had it been possible to make our measurements instantaneously, this condition might perhaps have been fulfilled. In practice it was unattainable. As measured by our relatively time-consuming procedure, loss of arginase activity in the presence of salicylate at 37° continued (although at an ever diminishing specific rate) for an indefinite period (with moderate concentrations for at least 24 hours), and did not cease until the enzyme had become all but completely inactive. Under these circumstances no better expedient presented itself than to measure, as we did, the action over the first 30 minutes of contact with the denaturant. Such action, it is evident, will not correspond to activity at any one determinable instant, but may at the best represent approximately the mean activity over the whole period of measurement.

In spite of the element of uncertainty thus inherent in the method we believe that the reported activities reflect with sufficient accuracy the *relative* effects of different concentrations of each denaturant. This belief is supported by the fact, already mentioned, that with the technique adopted the relation between quantity of enzyme and action in 30 minutes is found to take exactly the same form in the presence as in the absence of salicylate. This would imply that the fraction of enzyme inactivated is independent of its total amount, which is just what the known phenomena of denaturation (5) would have brought one to anticipate. That in this respect our method leads to the theoretically predictable result would seem to justify a certain confidence in its other indications. This confidence is increased by the general similarity of our curves to that of Anson and Mirsky.

All four of the curves are in fact S-shaped, though not all have exactly the same form. Those for salicylate and thiocyanate (Fig 1) are nearly symmetrical, with points of inflection at about 50 per cent inactivation. Comparison of the salicylate curve with that of Anson and Mirsky (1) shows that, in spite of a slight difference in form and a great difference in the range of concentrations covered (hemoglobin was completely denatured by 0.5 M salicylate), the two curves have a striking resemblance. It seems reasonable to infer that the process of inactivation by salicylate is largely, if perhaps not wholly, identical with that of denaturation. Thiocyanate, it is clear, acts in precisely the same way as salicylate, but, molecule for molecule, is less than half as effective. Guanidine hydrochloride gives a curve (Fig 2), which in its initial segment practically coincides with that for salicylate, but at concentrations above 0.55 M, at which about one-third of the enzyme has been inactivated, guanidine becomes less effective than

salicylate Its curve as a whole is accordingly asymmetrical The Duponol curve (Fig 3) departs still further from the salicylate pattern Of the four inactivating agents under consideration Duponol is, moreover, incomparably the most effective A concentration as low as 0 0001 M (0 003 per cent) suffices to produce a detectable loss of arginase activity, while at 0 00065 M the loss has reached 50 per cent, and at 0 002 M 93 per cent This is entirely in consonance with the known efficacy of long chain alkyl sulfates as protein denaturants Anson (6), for instance, found that hemoglobin is rapidly denatured by 0 0008 M Duponol PC (a mixture of alkyl sulfates with from 12 to 18 carbon atoms)

Let it be assumed that inactivation by denaturants is a reversible phenomenon, that it is effected either by chemical combination with or physical alteration of the enzyme, and that in either case it can be represented by the equation  $E + nD \rightleftharpoons ED_n$ , in which  $E$  is the enzyme,  $D$  is the denaturant, and  $n$  is the number of molecules of the latter required to convert 1 molecule of the former into the inactive form  $ED_n$  Then in the usual case, in which specific enzyme concentration (7) is small and combined denaturant therefore negligible, the equilibrium state should be described by the equation

$$\frac{\alpha}{1 - \alpha} D^n = K$$

in which  $\alpha$  is the fraction of enzyme in the native or active state,  $1 - \alpha$  is accordingly the fraction inactivated or denatured,  $D$  is the total molar concentration of the denaturant, and  $K$  is a constant In any case to which the equation applies, a plot of  $\log [\alpha/(1 - \alpha)]$  against  $\log D$  will be a straight line of slope  $n$  cutting the axis of  $\log [\alpha/(1 - \alpha)]$  at  $\log K$  When  $n$  and  $K$  are known, fractional inactivation,  $1 - \alpha$ , can be calculated as  $D^n/(D^n + K)$

In the particular case of Duponol C,  $\log [\alpha/(1 - \alpha)]$  plotted against  $\log D$  does actually yield a straight line, from which only two of the points show a sensible deviation These two are the first ( $1 - \alpha = 0 02$ ), to which, since its determination is subject to a very large percentage error, little weight need be given, and the last ( $1 - \alpha = 0 928$ ) The (very slightly adjusted) line drawn through the remaining points has a slope of 3, and indicates for  $K$  a value of  $2 63 \times 10^{-10}$  The curve of Fig 3 is drawn in conformity with these values and it will be seen that, up to a Duponol concentration of 0 0001 M and a fractional inactivation of 0 8, it fits the experimental points almost perfectly Except then, for the highest degrees of inactivation, the data agree with the postulates upon which the general equation is based, and indicate, these postulates granted, that,

at pH 8.4, 3 molecules of sodium dodecyl sulfate are concerned in the denaturation of each molecule (or each active center) of arginase. Whether the  $ED_3$  complex, the existence of which is thus indicated, corresponds in any sense to either of the two stoichiometric albumin-dodecyl sulfate complexes predicated (at pH 6.8) by Putnam and Neurath (8), it is not possible to say. At Duponol concentrations of 0.002 and 0.004 M (the latter not represented in Fig. 3) the effects are less than the theory would predict, but it may be pertinent to remember in this connection that Duponol C is not a perfectly pure product.

With guanidine the graph of  $\log [\alpha/(1 - \alpha)]$  against  $\log D$  is a line, which, although straight for fractional inactivations up to 0.76, becomes thereafter definitely curved. The straight portion of the line has a slope of almost exactly 2.5, the value indicated for  $K$  being 0.451. The data for thiocyanate and salicylate show the same type of departure from the Duponol pattern, but in an exaggerated form. With the former, the straight part of the line extends only to  $1 - \alpha = 0.45$ , with the latter only to 0.35. In each case this straight part has a slope, as with guanidine, of 2.5, while its intercept would make  $K$  for thiocyanate 2.72, and for salicylate 0.427 (almost the same as for guanidine). A slope of 2.5, if it has any significance at all here, is probably to be interpreted as an average and as indicating the existence of more than one type of combination, and the consequent establishment of more than one equilibrium, between enzyme and denaturant (9).

With use of the  $n$  and  $K$  values just specified to calculate fractional inactivations, one obtains, for salicylate, thiocyanate, and guanidine hydrochloride respectively, the curves, which, in Figs. 1 and 2, beginning as continuous lines, are completed as broken ones. In their later segments the continuous lines are simply drawn through the experimental points. It will be seen that up to the point where the continuous and the broken lines diverge, observed and calculated results are in each case in entire accord. Beyond that point, coming earliest with salicylate and latest with guanidine, each denaturant shows a greater (not, as with Duponol, a smaller) effect than the equation used would predict.

Anson and Mirsky (1) interpreted their curve in terms of a complicated equilibrium, involving the reaction of 1 molecule of hemoglobin with a considerable number of molecules of salicylate, a reaction resulting in the formation, by successive stages, of a whole series of hemoglobin-salicylate compounds. Our own curves might doubtless be interpreted in a similar sense, but the relations set forth in the preceding paragraphs prompt us to suggest, as a speculative possibility, that in each of the graphs the observed loss of activity is really the summation of two separate effects. The first

of these is the reversible denaturation, the second, independent thereof, is a directly depressant action of the denaturing salt upon the native and still active fraction of the enzyme. The first is governed by the equilibrium  $D^n \alpha / (1 - \alpha) = K$ , and is operative at all concentrations of the denaturant, the second comes into play only at concentrations above a certain rather high level. The first is represented in Figs. 1 and 2 by the curves which terminate as broken lines, the second by the intervals between the broken lines and the continuous ones. With Duponol concentrations remain throughout so low that the second effect never makes an appearance.

For the actual existence of the postulated double mechanism we have no direct evidence. It is well known, however, that unduly concentrated media are in general unfavorable to the action of enzymes. We have found a pertinent example in the action upon arginase of NaCl, with which denaturation is not a factor. Applied under the same conditions as the denaturants, NaCl had little effect upon arginase activity in concentrations up to 1.67 M, but reduced it by half when the concentration was raised to 3.34 M.

Even if the hypothesis here advanced is correct, the indicated  $K$  values cannot, for reasons already discussed, be regarded as other than rough approximations to the true equilibrium constants. Such as they are, however, they would suffice to indicate that the affinities of salicylate and guanidine hydrochloride for arginase are about equal, while that of thiocyanate is considerably less, and that of sodium dodecyl sulfate out of all comparison greater.

#### SUMMARY

The enzyme arginase is inactivated by each of the protein denaturants, sodium salicylate, potassium thiocyanate, guanidine hydrochloride, and sodium dodecyl sulfate (Duponol C). Of these four, thiocyanate is the least, and Duponol C incomparably the most effective. In all cases the curve relating fractional inactivation to concentration of denaturant is S-shaped, resembling more or less closely the curve relating salicylate concentration to percentage denaturation of hemoglobin (Anson and Mirsky). The curves for salicylate and thiocyanate are symmetrical, the others show different degrees of asymmetry. With Duponol C, the results can be interpreted to indicate that the sole factor depressing activity is denaturation, and that this involves the interaction of 3 molecules of dodecyl sulfate with 1 of the enzyme. With the other three denaturants, the situation cannot be formulated so simply, and certain of its features suggest that the effects of denaturation may here be supplemented by a directly depressant effect of the denaturing salt upon the undenatured fraction of the enzyme.

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# THE FLUOROPHOTOMETRIC DETERMINATION OF URANIUM IN BIOLOGICAL MATERIAL\*

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For a comprehensive study of the excretion and distribution of uranium in animals, a sensitive and accurate analytical procedure for the determination of uranium in biological material was necessary. Among the existing methods were a permanganate titration (1), a magneto-optic procedure (2), a radioactivity method (3), and the fluorescent technique of Hoffmann (4).

The fluorescent method was chosen for further study because it offered three advantages: specificity, sensitivity, and economy of equipment. In addition, Bloor's<sup>1</sup> investigations had indicated that the accuracy of the fluorescent techniques of Hoffmann (4) could be improved.

Briefly, Bloor's procedure was as follows. The uranium sample was fused with sodium fluoride in a platinum dish, the fusion illuminated with ultraviolet light, and the intensity of fluorescence determined by measuring the depth of a methylene blue solution required (Duboscq colorimeter) to absorb the emitted light. Since the sample was compared with a blank in total darkness, it was necessary that the investigator's eyes be dark-adapted.

To facilitate the measurement of fluorescent intensities and to increase the accuracy of the measurement, a fluorophotometer was constructed. This instrument permitted the accurate analysis of quantities of uranium as low as 0.0005  $\gamma$ . However, the presence of even small amounts of extraneous inorganic material was found to give erroneous results, usually by quenching the fluorescence (5). Therefore, it became necessary to devise a procedure for the isolation of uranium to make the method applicable to biological specimens.

The affinity of protein for uranium, even at its isoelectric point, served as the basis of the isolation procedure developed. In conjunction with electrolysis for the removal of heavy elements other than uranium, the isolation procedure permitted the accurate analysis of uranium in concentrations less than 1 part per million of fresh tissue by fluorophotometry.

\* This paper is based on work performed under contract No. W-7401-Eng-49 for the Manhattan Project at the University of Rochester.

<sup>1</sup> Bloor, W. R., unpublished results.



## EXPERIMENTAL

*Fluorometry*

*Fluorophotometer*—Recently, while this report was in preparation, a report appeared (6) describing an apparatus similar in many respects to that presented here. The instrument and procedure (6), however, lack the sensitivity and precision required for biological study.

*Sample Holder*—Bloor's technique<sup>1</sup> of fusing the uranium-sodium fluoride flux in platinum foils was adopted. The sample cup was made of 0.13 mm (0.005 inch) platinum foil, 20 by 24 mm, having a circular indenta-

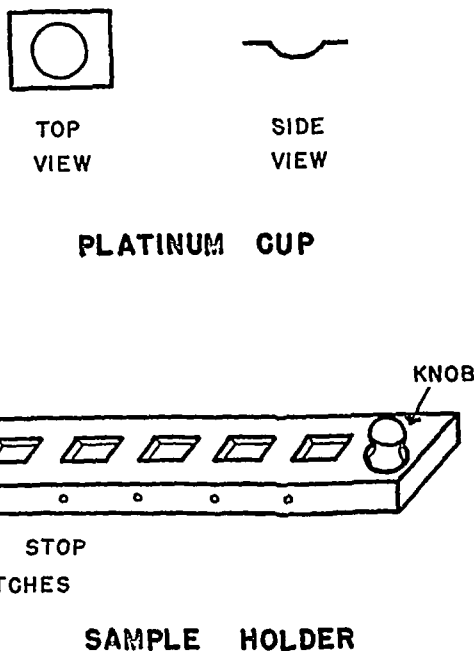


FIG 1 Perspective drawings of the platinum fusion cup and sliding brass sample holder

tion 11 mm in diameter and approximately 1.5 mm deep at the center, as indicated in Fig 1. In the fluorophotometer, the foils were brought into reading position by means of a sliding brass bar (Fig 1) which accommodated five samples and was equipped with a spring stop mechanism to facilitate centering of the specimens.

*Illumination*—Several types of ultraviolet sources were tested: General Electric bulb BH4, Mineralight model V41, a mercury arc, and others. All of these activated the uranium-sodium fluoride flux to a bright, yellow-green fluorescence, indicating that the wave-length of the ultraviolet radiation was not critical. For the fluorophotometer, the Conti-Glo lamp (Conti-

mental Lithograph Company, Cleveland) equipped with a clear glass bulb (General Electric, AH4) was the most satisfactory. To minimize variations in radiation output, the lamp was supplied with a constant voltage transformer (Sola, model 30808). Corning Filter 5874 (heat-resistant) and several thicknesses of Filter 9863 were interposed between the lamp and sample holder to remove visible radiation. A cooling system, a fan or water jacket, removed the heat generated by this lamp.

*Photometer*—The wave-length of the emitted fluorescent light has been reported (5) to be principally  $555\text{ m}\mu$ . To isolate this wave-length, Corning Filters 3484 and 9780 were placed above the illuminated sample. Above these filters was fixed a light-tight box housing the phototube, as indicated in Fig. 2. The current of the phototube, after amplification, operated a

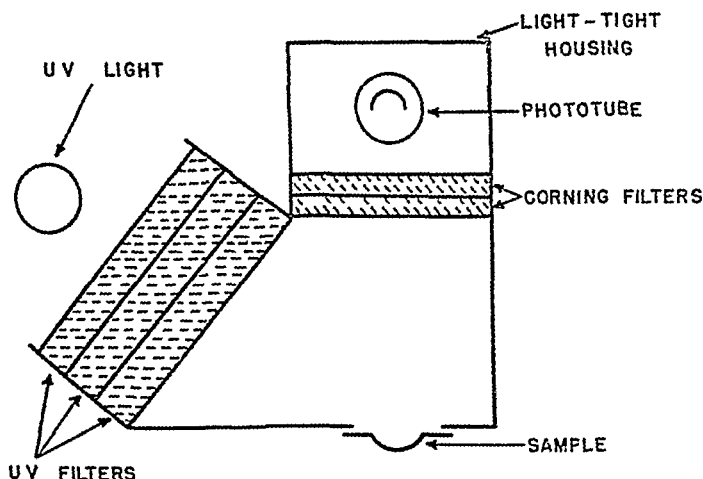


FIG. 2 Section drawing of the fluorescence chamber. For details, refer to the text.

galvanometer. The wiring diagram of the amplifier is given in Fig. 3. It is a slight modification of a standard d.c. amplifier circuit (7). By the use of three separate grid resistors of different resistances and an optional galvanometer shunt, the analytical range could be varied 1000-fold. Since the phototube currents were very small, the stability was improved by keeping the connections to the control grid of the amplifier tube as short as possible. Accordingly, the amplifying tube and grid resistors were mounted in the phototube housing. It was necessary to keep a fresh supply of silica gel in the chamber to avoid instability caused by humidity. In operation, the circuit was adjusted to give a 0.6 volt drop across the filament and a plate current of 100 microamperes.

*Procedure*—Because of the small amounts of material taken for analysis,

all samples were prepared in solution form, adjusted to a range in concentration of uranium from 0.01 to 20  $\gamma$  per ml. To prevent the adsorption of the small quantities of uranium on the walls of vessels, etc., the solutions were made at least 0.1 N with respect to free nitric acid.

A 0.1 ml aliquot of the solution to be analyzed was pipetted into a platinum cup. The cup was placed on an asbestos board and the contents evaporated to dryness on an electric hot-plate. If organic material was present, the temperature was raised until charring took place. The asbestos board and cup were then removed and allowed to cool. Approx-

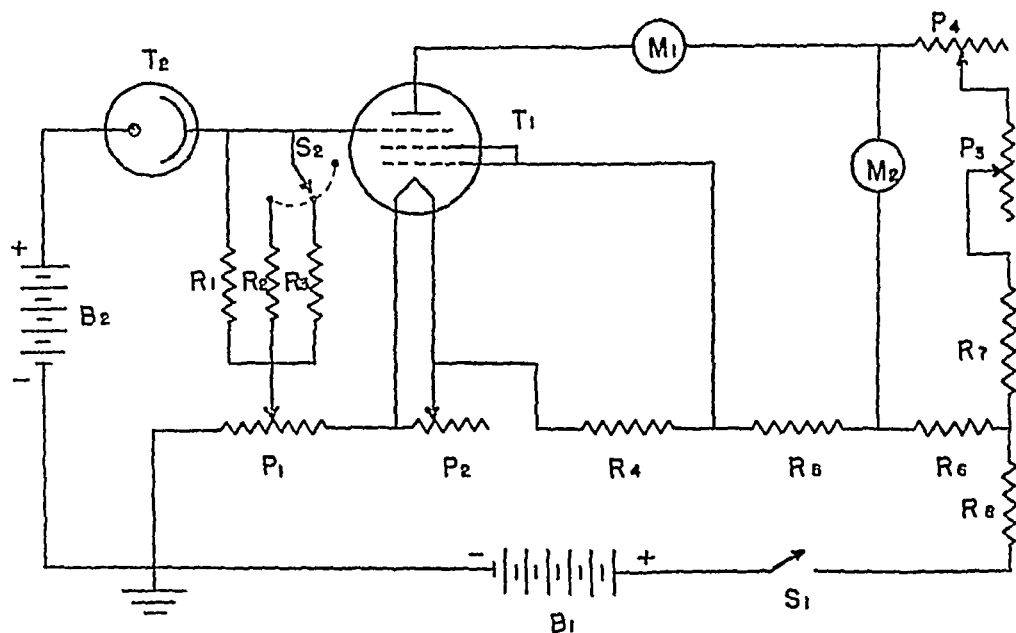


FIG 3 Wiring diagram of amplifier circuit.  $T_1$ , No. 1LN5,  $T_2$ , No. 929, base removed,  $B_1$ , 24 volt storage battery,  $B_2$ , 22.5 volt B battery,  $S_1$ , single pole, single throw toggle switch,  $S_2$ , special switch,  $M_1$ , 200 microampere meter,  $M_2$ , galvanometer, 6 microamperes full scale,  $P_1$ , 60 ohms,  $P_2$ , 200 ohms,  $P_3$ , 10,000 ohms,  $P_4$ , 1000 ohms (wire-wound),  $R_1$ , 100,000 megaohms,  $R_2$ , 10,000 megaohms,  $R_3$ , 1000 megaohms,  $R_4$ , 200 ohms,  $R_5$ , 82 ohms,  $R_6$ , 5.6 ohms,  $R_7$ , 1000 ohms,  $R_8$ , 220 ohms.

mately 80 mg of sodium fluoride (Baker and Adamson, reagent grade) were added to the cup and the sample twice fused and read in the fluorophotometer.

**Fusion**—The reading given by a known quantity of uranium was relatively unaffected by some variation in the amount of sodium fluoride employed which made it convenient to measure the fluoride by means of a small scoop which delivered  $80 \pm 20$  mg. This quantity gave best results with the size of the platinum cup previously described. Two samples were fused simultaneously by placing them side by side on a nichrome wire rack above a Meker burner.

The use of a large diameter pipe (1 5 inch) connected directly to the gas main, or to a constant pressure head, obviated changes in the gas pressure, which insured an even fusion temperature

*Reading*—All samples were analyzed in quadruplicate. A convenient set would, for example, consist of

1 blank	× 4 =	4
3 standards	× 4 =	12
20 samples	× 4 =	80
		—
		96 fusion cups

Such a test took one operator 6 to 7 hours working time

Before a series of readings, the fluorophotometer was given about  $\frac{1}{2}$  hour to reach thermal equilibrium. By adjustment of the zero controls, the galvanometer was set to give a reading of 10 for the first blank. This blank was kept in the sample holder and used as a reference point for the reading of all subsequent samples.

*Contamination Control*—To analyze extremely small quantities of uranium, it was necessary to exercise a rigorous control over operating conditions. No dry uranium compounds were permitted in the analytical room. All glassware for quantitative use was left overnight in cleaning solution and, immediately before use, rinsed thoroughly in 1 per cent nitric acid followed with distilled water. The platinum cups were boiled in 1 per cent nitric acid and rinsed several times with distilled water, reshaped, and kept under distilled water until ready for use. Immediately before the samples were to be analyzed, the cups were again boiled with dilute nitric acid and rinsed several times with distilled water. Extreme care was exercised during the period in which samples were pipetted, dried, and fused.

*Results*—A linear relationship between galvanometer deflection and the quantity of uranium present was observed throughout the analytical range (0.001 to 3.0  $\gamma$ ).

*Analysis of Pure Solutions of Uranyl Nitrate*—To obtain a statistical analysis of the accuracy of the method, a series of unknowns was prepared by a disinterested person and analyzed in the routine manner. The results of this study are presented in Table I.

It was shown that the absolute error of the method increased with increasing quantities of uranium, giving a fairly consistent per cent error. Because of this fact, and because of convenience of the term "per cent error," the standard deviation was calculated as a percentage. The results of the statistical analysis of the data in Table I are presented in Table II.

It can be seen from Table II that the standard error of the fluorophoto-

metric method, with pure solutions of uranium, is less than 3 per cent. Of twenty-seven samples analyzed, the largest error observed was 10 per cent.

*Analysis of Tissue Specimens*—Poor recoveries were observed when known quantities of uranium were placed in porcelain crucibles. Accordingly, platinum ware was employed. All tissue specimens were dry-ashed in a muffle furnace at temperatures not exceeding 750°. At lower temperatures, ashing was incomplete, at higher temperatures, the ash of some tissues (urine especially) tended to crawl out of the crucibles.

The ash of all tissues reduced the intensity of the fluorescence of uranium. Typical of the results obtained are the data presented in Table III in which

TABLE I  
*Results of Fluorophotometric Analysis of Uranyl Nitrate Solutions*

Contained	Found	Contained	Found
$\gamma$	$\gamma$	$\gamma$	$\gamma$
0.228	0.238	0.040	0.040
0.228	0.229	0.040	0.042
0.228	0.229	0.040	0.042
0.228	0.225	0.0228	0.021
0.170	0.170	0.0228	0.023
0.170	0.174	0.0228	0.023
0.170	0.175	0.0228	0.023
0.170	0.170	0.020	0.020
0.160	0.159	0.020	0.020
0.160	0.152	0.020	0.020
0.160	0.163	0.010	0.011
0.080	0.083	0.010	0.011
0.080	0.078	0.000	0.000
0.080	0.077	0.000	0.002
0.080	0.079	0.000	0.000

fluorescence was reduced approximately 20 per cent by microgram quantities of either bone ash or ferric iron.

When applied to the analysis of biological samples, a 17 per cent inhibition was observed when the concentration of uranium in bone ash was 2 parts per thousand. Obviously, to determine uranium in lower concentrations (1 p.p.m.), some method for the isolation of uranium was necessary.

#### *Isolation of Uranium from Biological Material*

The observations of Dounce<sup>2</sup> that uranium forms a stable complex with proteins served as the basis for further experimentation, which led to the development of the isolation procedure described below.

<sup>2</sup> Dounce, A. L., unpublished results.

*Procedure*—The tissue ash was dissolved in a minimum of 2 N hydrochloric acid and an aliquot containing 50 to 100 mg of ash transferred to a centrifuge tube graduated at 10 and 30 ml. 2 drops of brom-cresol green indicator were added and the solution neutralized to pH 4.5 (green color) with 1 N sodium hydroxide. 3 ml of 1 N sodium acetate-acetic acid buffer (pH 4.5) and then a solution containing 100 mg of one of the protein preparations described below were added. The resultant solution was diluted to 30 ml and, after thorough mixing, the tube was immersed in a hot water bath at 80° for 45 minutes. The coagulum which formed was separated by centrifugation, washed once with fresh 0.1 N acetate buffer

TABLE II  
*Statistical Analysis of Accuracy of Fluorophotometric Method*

No. of samples	27
Concentration range $\mu\text{g./ml.}$	0.1–2.9
Coefficient of correlation	0.999
Range of recovery	<i>per cent</i> 92–110
Mean recovery	101
Standard deviation	3.9
“ error	2.6

TABLE III  
*Inhibition of Uranium Fluorescence*

Bone ash, 0.2 $\gamma$ U per sample		Ferre chloride 0.1 $\gamma$ U per sample	
Added	Inhibition	Added	Inhibition
$\gamma$	<i>per cent</i>	$\gamma$	<i>per cent</i>
0.0	0	0.0	0
0.1	0	0.4	5
1.0	5	0.8	12
10.0	11	4.0	25
100.0	17		

(pH 4.5), and again centrifuged. The washed precipitate was dissolved in concentrated nitric acid and made up to a volume of 10 ml. 0.1 ml. aliquots were then taken for fluorophotometric analysis. “Blank” determinations were carried through the procedure with each set of samples as a check against contamination of reagents.

*Effect of Protein Concentration*—In the preliminary studies, crystalline egg albumin was employed. Because of the expense and trouble involved in its preparation, the crystalline protein was discarded in favor of commercial egg albumin, precipitated twice with ammonium sulfate, and dialyzed free of salt. Later, it was found that a preparation of bovine al-

bumin (Fraction V from bovine plasma, Armour Laboratories, Chicago) could be used dnrectly without preliminary purification or manipulation

In Table IV are presented the results of two experiments designed to determine the effect of varying quantities of protein on the recovery of

TABLE IV  
Effect of Varying Protein Concentration on Recovery of 10  $\gamma$  of Uranium

Concentration of protein  mg per ml	Recovery	
	Test 1 per cent	Test 2 per cent
0 63	62	64
0 85	72	78
1 25	86	83
1 58	88	93
1 88	91	92
2 00	89	
2 50	94	104
3 00	102	100

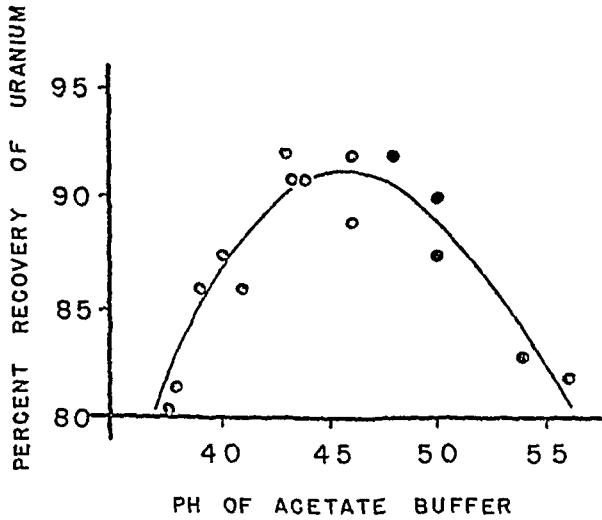


FIG 4 Effect of pH on the precipitation of the uranium-protein complex

uranium It is evident that, with the volumes employed, the protein concentration should be in excess of 2 5 mg per ml

Effect of pH—Below pH 4 0 and above pH 5 0 it is difficult to obtain the complete coagulation of the protein which is essential for good recoveries of uranium In Fig 4 are presented the results of studies designed to determine the optimal pH for recovery of uranium These data indicate

that pH 4.5 is optimal, but a change of  $\pm 0.2$  pH unit does not seriously reduce the recovery.

**Solubility of Ash**—Bone ash was assumed to be the least soluble ash encountered in biological material. Accordingly, "solubility" experiments were conducted to determine the maximum quantity of bone ash soluble in acetate buffer at pH 4.5. This was found to be approximately 4.0 mg per ml. However, as is indicated in Table V, heating, which was necessary to coagulate the protein, reduced the "solubility" to 3.3 mg per ml. Since a volume of 30 ml. was customarily used, the maximum quantity of ash taken for analysis was 100 mg.

**Efficiency of Isolation**—An experiment was conducted to determine how completely uranium was separated from tissue ash and to determine how much tissue ash carried through the isolation. The standard procedure

TABLE V

*Effect of Temperature on Solubility of Bone Salt*

— denotes complete "solubility," + denotes precipitation

Time	Temperature	Concentration of bone salt			
		3.3 mg per ml	3.5 mg per ml	3.8 mg per ml	4.0 mg per ml
<i>min</i>	<i>°C</i>				
0	55	—	—	—	—
18	60	—	—	—	—
22	65	—	—	—	—
28	70	—	—	—	—
36	76	—	—	—	+
43	80	—	+	+	+
73	90	+	+	++	+++

was applied to two solutions of pure uranyl nitrate (10  $\gamma$ ) and to six solutions to which 100 mg. of bone ash had been added. The ash content of the resulting washed protein precipitates was determined. In the experimental samples, containing 100 mg. of bone ash, 4.4, 4.1, 4.4, 3.9, 4.0, and 3.7 mg. (average 4.1 mg.) of ash were found, and in the control samples, containing no bone ash, 5.4 and 5.6 mg. (average 5.5). These results indicated that the ash content of the uranium-protein complex is less in the presence of bone salt than in its absence. This seemingly paradoxical result is best explained by the fact that, in the presence of bone ash, the protein coagulum is more dense and occupies a smaller volume than when salt is absent (familiar phenomenon of the salting-out of proteins). Consequently, a smaller volume of wash solution containing 8 mg. per ml. of sodium acetate was retained by the protein coagulum when



bone salt was present Under the circumstances, it seems likely that very little, if any, bone ash carries through the isolation procedure

TABLE VI  
*Results Obtained with Protein Isolation*

	Bone samples	Pure solutions	Soft tissues
No. of samples	16	16	28
Concentration range, $\mu$ g m	1-1000	0.01-10	0.5-50
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Mean recovery	96	92	90
Standard deviation	9.1	7.0	5.8
Range of recovery	70-104	77-102	83-105

TABLE VII  
*Recoveries of Uranium from Liver, Spleen, and Blood Both by Electrolysis and by Protein Isolation*

No. of samples	17
Concentration range, $\mu$ g m	2-25
	<i>per cent</i>
Mean recovery	92.5
Standard deviation	7.1
Range of recovery	75-103

TABLE VIII  
*Over-All Summary of Analytical Results*

	Pure solutions	Tissues
No. of samples	16	61
Concentration range, $\mu$ g m	0.01-10	0.5-1000
	<i>per cent</i>	<i>per cent</i>
Mean recovery	92.0	92.3
Standard deviation	7.0	7.0
Range of recovery	70-102	70-105

*Results*—The results obtained from the analysis of pure solutions of uranium and of samples of bone and soft tissue<sup>3</sup> to which known quantities of uranium were added are presented in Table VI

Not included in Table VI are the results obtained with liver, blood, and

<sup>3</sup> The following tissues were taken: kidney, bladder, genitals, heart, lung, stomach and contents, intestines, leg muscle, and skin and hair

spleen, from which recoveries as low as 38 per cent were observed. This difficulty was overcome by electrolyzing such samples in a small mercury cathode cell prior to isolation of the uranium by the protein method.

To the HCl-ash solution, perchloric acid was added to give a final concentration of 3 per cent and current (10 volts, d c) passed through for 1 hour. The electrolyzed solutions were then subjected to the isolation procedure described above.

With the electrolysis, good recoveries were obtained from liver, spleen, and blood, as indicated in Table VII.

The over-all average recoveries are presented in Table VIII. It is clearly evident from these data that uranium can be recovered from tissue equally well as from distilled water. Although the recoveries were not strictly quantitative (92 per cent), they were consistent (standard deviation, 7 per cent) and within the accepted limits of biological variation.

#### DISCUSSION

The fluorophotometric method for the determination of uranium offers extraordinary specificity, sensitivity, and, for the quantities analyzed, unusual accuracy. The ultimate limit of sensitivity has not been determined. If higher value grid resistors are employed, the fluorophotometer can be adjusted so that uranium can be detected in even smaller quantities than reported above. In our experience, slight impurities in the best grade of reagent sodium fluoride set a lower detection limit of 0.0005  $\gamma$  of uranium per fusion cup. Translated in terms of tissue, this is equivalent to 0.005  $\gamma$  per gm. of fresh, soft tissue.

The use of organic reagents in microanalytical procedures is gaining in popularity. Proteins, however, represent a group of organic reagents rarely employed. The point of interest in this particular application is the fact that the combination with uranium is practically quantitative at the isoelectric point. Good evidence<sup>2</sup> has been obtained that it is a direct combination with protein and *not* the usually observed coprecipitation phenomenon. Since, at the isoelectric point, electrovalent combinations with both cations and anions are minimal, it is probable that the uranium-protein complex which forms is a covalent type of combination. It is possible that the technique employed in the development of the isolation procedure may be applied to (a) the isolation of other metals and (b) the evaluation of the type of linkage between protein and various ions.

#### SUMMARY

With a protein isolation, and with electrolysis when necessary, uranium can be quantitatively determined fluorophotometrically in tissue samples in concentrations as low as 0.005  $\gamma$  per gm. of soft tissue.

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# THE RELEASE OF THE SULFUR FROM THE TISSUES OF RATS FED LABELED METHIONINE\*

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Since the pioneer investigations of Schoenheimer and coworkers with both ammonia labeled with heavy nitrogen and amino acids labeled with either deuterium or nitrogen, it has been evident that the amino acid components of many of the tissue proteins readily interchange with the free amino acids of the protoplasm. However, not all tissue proteins behave similarly with regard to the uptake and release of labeled amino acids. Some tissues such as intestinal mucosa and kidney are particularly active in this respect. Other tissues such as brain and muscle are much less active. It therefore becomes necessary to investigate this phenomenon in detail in order to gain some insight into the factors which control the rates of incorporation and release of the amino acids in the protein of different tissues and of the individual proteins of any one tissue.

Such studies have already been initiated in a number of laboratories. In 1944 Shemin and Rittenberg (1) reported on the results of an investigation into the release of glycine labeled with heavy nitrogen from the tissue proteins of rats. The most important finding in this work was that the nitrogen once "fixed" into the animals' tissues was but slowly released into the urine. There occurred in the animals an equilibration of the nitrogen so that all products approached an "average isotope concentration." In studies of a more restricted nature, the behavior of the antibody proteins has been followed with glycine and tyrosine labeled with heavy nitrogen (2). Thus it was observed that the behavior of the antibody in passive and active immunity is entirely different from a metabolic point of view. The behavior of plasma protein labeled with heavy nitrogen in the  $\epsilon$  group of lysine has been followed by feeding large doses of labeled lysine to a donor dog and then injecting the plasma from the donor animal into recipients (3).

The work reported here is of a nature similar to that of Shemin and Rittenberg. Methionine labeled with radioactive sulfur was fed to rats,

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and the tissues were examined after various intervals for the presence of the label in the protein fraction. From the results obtained, there is evident the same tendency for the isotope concentration to approach an approximately constant level, with a small loss into the urine. Consequently, the approach to an "average isotope concentration" observed by Shemin and Rittenberg is not due to any special propensity of nitrogen to be reincorporated into carbon residues, but is a more general phenomenon concerned with the metabolism of protein.

### Methods

*Experimental Animals*—The experiments were done on a series of thirty-four rats divided into six groups. The animals were mixed male and female, 3 or 4 months old, but of widely varying weights (175 to 389 gm.)

TABLE I  
*Summary of Experimental Data*

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
No of rats	9	9	4	5	4	3
Duration of experiment, days	1	14	2	10 2	4	7
Methionine fed, mg	1 34	1 34	2 68	2 68	3 35	3 35
" " counts per min	5,770	5,770	11,500	11,500	14,400	14,400
No of washes*	4	2	4	3	3	3

\* Trichloroacetic acid washes of rat proteins

They were fed a standard diet<sup>1</sup> for a period of 7 days before the experiments and during the whole time were maintained at 22–23°.

*Treatment of Animals*—The animals were fed methionine labeled with radioactive sulfur (4) by stomach tube in the amounts indicated in Table I, dissolved in 1 ml of water. After the number of days indicated (Table I) the animals were killed by withdrawing blood from the heart into oxalate. This was done under ether or nembutal, neither of which appeared to affect the results.

*Preparation of Samples*—Either the tissues were removed from the animals immediately and minced or homogenized in a Waring blender

<sup>1</sup> The diet had the following composition: casein 150 gm, fat 140 gm, salt mixture 55 gm, sugar 655 gm, cod liver oil 20 ml, wheat germ oil 12 ml, choline chloride 0.5 gm, thiamine 3 mg, riboflavin 4 mg, pyridoxine 3 mg, calcium pantothenate 30 mg, nicotinamide 10 mg, inositol 200 mg, *p*-aminobenzoic acid 5 mg. The salt mixture is that of McCollum and Davis as modified by Tufts and Greenberg (*J Biol Chem*, 122, 693 (1937–1938) (Mixture 3)).

or the whole animal was frozen in a dry ice-acetone mixture and kept in the refrigerator until dealt with (not more than 2 days). The tissue preparations were precipitated with trichloroacetic acid and washed several times with the same acid (Table I).

The protein precipitates were digested with the Pirie (5) reagent and determination of total sulfur and radioactivity was made as described in a previous publication (6). In Groups 1 and 2 the liver protein was divided into equal portions, one of which was used for the determination of the total sulfur of the material, and the other was subjected to the differential oxidation procedure of Evans (7). This results in the oxidation of the cystine to sulfate but leaves the methionine unaffected. The sulfate was precipitated with benzidine after removing the nitric acid, and the sulfur and radioactivity determined. Methionine in the filtrate from the benzidine sulfate was oxidized with the Pirie reagent and sulfur and radioactivity determinations were again made. No great accuracy can be claimed for this method because of difficulty encountered in determining the end-point in titrating the benzidine sulfate from the cystine oxidation. There was contamination with a yellow color which made an exact determination of the salmon end-point very difficult in some instances. Also the presence of excess benzidine made the determination of methionine more difficult. In some instances sulfur was lost by ignition of the perchloric acid digests.

Total sulfur was determined in urine and feces samples by the same methods.

Some determinations of radioactivity were made on the trichloroacetic acid washes. These washes contained significant amounts of protein which were lost by decantation from the precipitates, and the results only served to indicate that the washes contained relatively little radioactive material in the group of animals sacrificed after 14 days from the time the methionine was fed.

*Expression of Results*—In a previous paper (6) we endeavored to compare the radioactivities in the sulfur fraction of animals of different weights fed different amounts of methionine by means of a formula giving a unit termed the standard replacement (S R). This was evolved from an earlier unit, per cent replacement (relative activity). Standard replacement was defined as follows:

$$S R = \frac{S A \text{ of sample} \times 10^3 \times W}{S A \text{ of methionine} \times C}$$

where  $W$  = weight of the animal in kilos. The specific activity (S A) of a sample is the radioactivity (in counts per minute) of the sample per milliequivalent of sulfur, and specific activity of methionine is the radio-

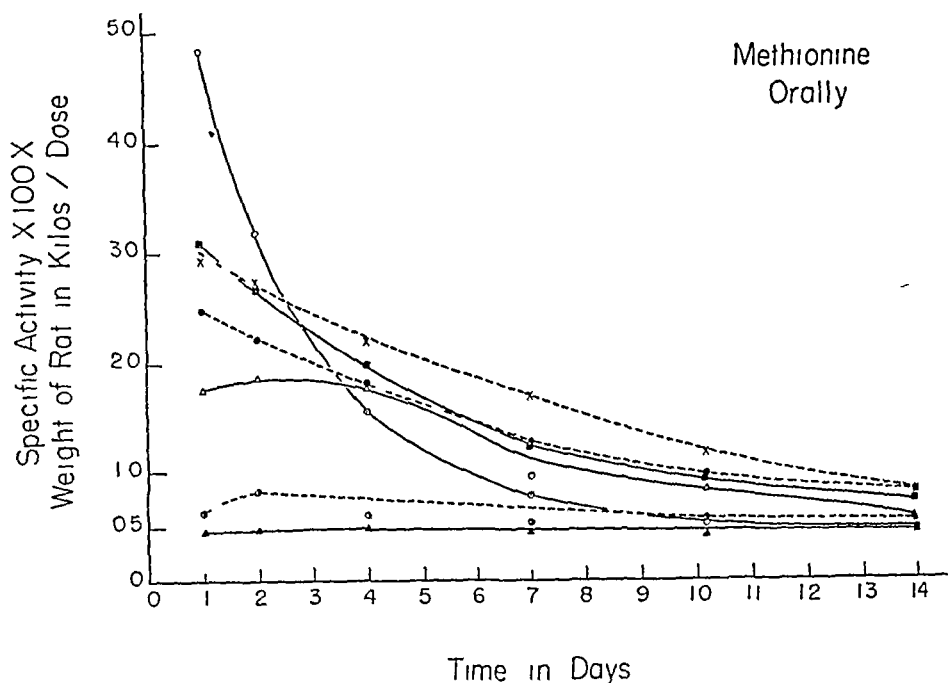


FIG 1 Rate of decrease in concentration of radioactive sulfur in the proteins of rat tissues labeled by feeding methionine containing radioactive sulfur O intestinal mucosa, ■ plasma, X kidney, ● liver, Δ spleen, ⊙ brain, ▲ residue (skin, hair, muscle)

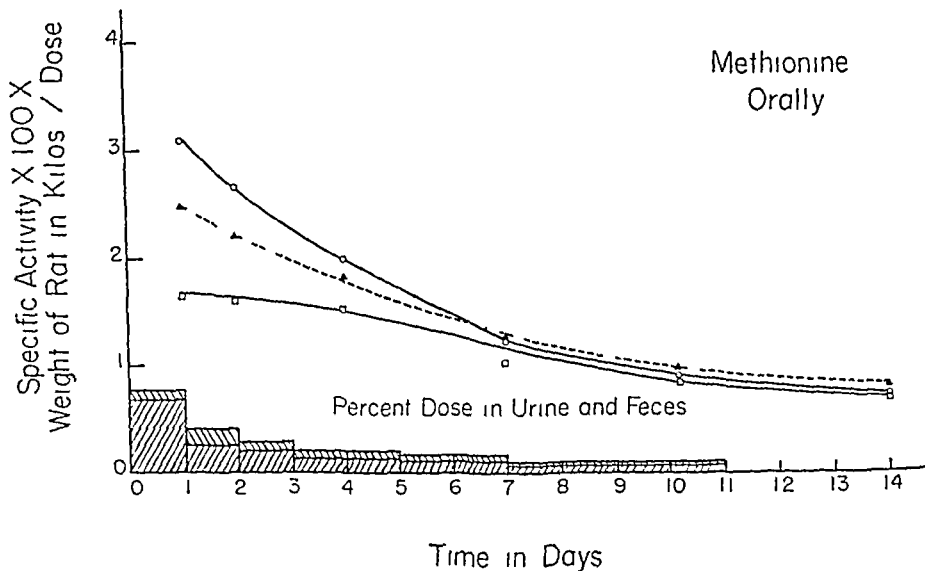


FIG 2 Rate of decrease in concentration of radioactive sulfur in cystine, methionine, and in the total sulfur of rat liver proteins labeled by feeding methionine containing radioactive sulfur, and per cent of the dose excreted in urine and feces The units on the ordinate are either standard specific activity (referring to the liver curves) or per cent (referring to the urine and feces excreted) O liver methionine, Δ liver total sulfur, □ liver cystine The upper section of cross-hatching represents feces, the lower urine

activity in the methionine administered ( $C$ ) per milliequivalent. When this formula is used, it is assumed that the specific activity of any given sample is proportional to  $C^2$ , and inversely proportional to the weight of the methionine in the dose and of the weight of the animal. But the treatment of the experimental data implied, and the doses of methionine used were such, that the specific activity of any sample was independent of the dose of methionine given. The methionine was a tracer dose. Also the specific activity of any sample is proportional to  $C$ , not to  $C^2$ . Consequently, it is necessary to abandon this method of expression and adopt one which will be referred to as *standard specific activity* ( $S S A$ ), where

$$S S A = \frac{S A \text{ of sample} \times 10^4 \times W}{C}$$

When this method of calculation is used, the results obtained with different species are brought into line.<sup>2</sup>

### Results

The main results are shown in Figs 1 and 2. The results for the urine sulfur given in Fig 2 are from the 1st day's urine of the rats of Groups 1 and 2. All other figures for urine excretion are from Group 2 only. Excretion of sulfur in the feces, shown in Fig 2, is from the rats in Group 2 only.

Results for urine and feces are expressed as per cent of the dose fed. All other results are in terms of standard specific activity.

The amount of radioactivity used in these experiments was much lower than that necessary to obtain accurate results, particularly for the less active tissues. For some tissues such as spleen it was necessary to combine samples from several rats in order to get significant values for the radioactivity. Determinations on the residues were performed in duplicate, but the radioactivities observed were so low that at times the agreement between the duplicate results was very poor. Had the necessary amounts of radioactivity been available, it would have been preferable to use 10 or more times as many counts per rat.

### DISCUSSION

It is seen from the results that the radioactivity in the residues of the rats stayed constant within the error of the determinations. These residues consisted of muscle, skin, hair, and bone, together with the remaining

<sup>2</sup> The work (6, 9) in which the erroneous expression was used, involved only methionine of the same specific activity, so the results are quite comparable within themselves and can be corrected if desired by multiplying by a factor ( $S A \text{ of methionine} \times 10^{-4} = 2.23$ ).



blood, and actually most of the radioactivity retained by the animals is present here. Therefore the specific activity of the residues is approximately the same as that of the average isotope concentration of the whole animal. According to Shemin and Rittenberg, the isotope concentration in every tissue protein should approach this value with time, from either direction. This assumes that the isotope in *all* tissues is in equilibrium with that in all others. However, the isotope once fixed in hair and skin ceases to be in equilibrium with that in the rest of the organism. Consequently, it may be considered that the isotope entering these tissues is lost in the same way as is isotope excreted into the urine. Therefore, because the isotope undoubtedly went into the hair (8), there must be so much less isotope capable of being exchanged with other tissues. There is no true constant average isotope concentration, but a steadily falling one. On this account no certain deductions can be made about the half life of any given tissue protein by using an average isotope concentration as reference. Such comparisons are of undoubted value but not as measures of true rates.

In the present experiments there is an additional difficulty in interpreting the rates. This is due to the conversion of methionine to cystine. As is shown in Fig. 2, the rate of fall of isotope concentration in the methionine of the liver protein is considerably greater than the rate of fall determined on the basis of total sulfur. The rate of fall of isotope concentration for cystine is correspondingly low. Previous studies have shown the conversion of methionine to cystine in tissues other than the liver (9), therefore similar situations must prevail elsewhere even if the cystine is not distributed to any great extent via the circulation. However, for comparative purposes between animals under different conditions, this probably does not seriously detract from the usefulness of such determinations.

The approach of the isotopic concentration in all tissues to an approximately constant value and the slow loss of isotope into the urine is established by both these results and those of Shemin and Rittenberg. *We fail to see where the results are in disagreement with the concept of endogenous metabolism as formulated by Folin (10)*. There is a slow *net* breakdown of tissue protein which is indicated in the isotope experiments by a loss of either  $N^{15}$  or  $S^{35}$  into the urine, just as in the data basic to the Folin theory there is a slow *net* breakdown of tissue protein indicated by a loss of creatinine and neutral sulfur into the urine. Indeed, isotope experiments can perhaps provide a direct measure of the endogenous protein metabolism, whereas the creatinine is only an indirect index of the same.

The term endogenous metabolism therefore remains useful as a description of the *net* results of the metabolism of protein and related substances.

This type of metabolism proceeds slowly, and it is quite obvious that it is impossible to explain the rapid uptake of isotopic amino acid by the proteins of tissues such as intestinal mucosa by the operation of this slow process. The Fohn deductions concerning protein breakdown *within the organism* are proved to be untenable by the isotope experiments.

The processes which result in the formation and breakdown of proteins within the organism and which must be responsible for the rapid uptake of amino acids have been referred to by Borsook and Keighley (11) as "continuing metabolism." Owing to the existence of this form of metabolism, isotopic amino acid is rapidly incorporated into the protein of many tissues. The continuing metabolism of such tissues is high, whereas that of certain other tissues, such as muscle, is low.

Following the incorporation of the amino acid into the rapidly metabolizing tissue proteins, there is a slower exchange of the amino acid in these proteins with the same amino acid in the tissue proteins of those tissues with low rates of continuing metabolism. In this process there is little loss of the amino acid or the products of its catabolism into the urine. Endogenous catabolism is slow.

Compared with the liver protein, the data show that the concentration of isotope 1 day after feeding methionine is greater in the plasma than in the liver. But the major part of the plasma protein is formed in the liver (9), consequently the liver must have formed plasma protein and passed it into the circulation faster than it formed its own cellular protein. The livers were not perfused, so that the true difference in concentration must have been greater than those actually observed. Later it is seen that the situation is reversed. This must be due to loss of isotope from plasma protein into other tissues including the liver and into the urine as products of catabolism. Plasma proteins probably mediate the equilibration or interchange of amino acids between the proteins of tissues. If they were interchanged only through the mediation of the free amino acid of the plasma, then a greater daily loss of isotope into the urine would have been anticipated. That is, the endogenous catabolism would have been larger than that observed. As isotopic protein broke down, the isotopic amino acid passing into the plasma would be diluted with the same amino acid from the food and would be lost by the operation of the general catabolic process. Actually after the 1st day there is little loss of isotope into the urine, and the formation of plasma protein (and liver protein) may be considered to act as a buffer to prevent the loss of amino acid by the operation of the processes of exogenous metabolism. This process of catabolism may be brought to the fore by flooding the organism with amino acid, particularly a single amino acid, all of which cannot be synthesized into plasma protein.

The data also show the outstanding activity of intestinal mucosa and kidney in respect to the uptake and release of the label. Friedberg (12) has already interpreted the activity of the mucosa as being due to the rapid synthesis of protein by this tissue for the formation of the secretion poured into the gut. Since the high uptake is also observed when the compound is injected into rats or dogs (9) and in experiments with chopped tissue,<sup>3</sup> the explanation is probably correct, but the secretion of protein by the intestinal mucosa into the circulation is not excluded by any experimental work so far carried out. The question, therefore, remains as to whether these outstandingly active tissues such as intestinal mucosa and kidney contribute to the formation of blood protein to any significant extent.

Brain and spleen show an apparent inactivity with regard to the uptake of isotope, but this may be due as much to impermeability of the brain tissue to amino acid (13) as to slow rate of uptake of amino acid by the protein. The significance of the humps observed in these two curves on the 2nd day is doubtful. Here the data are from four rats only, and owing to the small amount of tissue concerned, the error in the determinations may be quite large.

The small loss of radioactivity into the urine and feces, even though half of the methionine fed was of the D configuration, is worthy of note. This should be contrasted with the results of Shemin and Rittenberg (1) and of Schoenheimer, Ratnei, and Rittenberg (14) who observed much larger amounts of isotopic nitrogen in the urine after feeding glycine and leucine. In part, these results must be attributed to the relatively higher doses of these amino acids fed compared with the tracer doses of the methionine used in these studies.

#### SUMMARY

1 When methionine labeled with radioactive sulfur is fed to rats, the concentration of radioactive sulfur after 24 hours is greatest in the proteins of the intestinal mucosa and decreases in the order of kidney, plasma, liver, spleen, and brain. The residue of skin, hair, and muscle has a very low concentration of radioactive sulfur.

2 During the following 14 days, most of the radioactivity is lost from the active tissues and the isotope concentration throughout the animal becomes more or less stabilized at a lower level.

3 About 7 per cent of the administered radioactivity appears in the 1st day's urine. Thereafter the output decreases until on the 9th day there is only about 0.5 per cent in the urine. The total excretion into the urine in the 14 day period is estimated to be less than 20 per cent of the dose.

4 The maximum output of about 2 per cent of the administered dose

<sup>3</sup> Kornblum, R., and Tarver, H., unpublished work.

appears in the feces on the 2nd day. In succeeding days the output decreases and the total excretion in the 14 day period is estimated to be about 8 per cent of the dose.

5 The rate of loss of the isotope from the methionine in the liver protein is greater than from the cystine in the same tissue. This complicates the interpretation of the data.

6 The relationship of these results to the theory of protein metabolism is discussed.

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# SIMPLIFIED CALCULATIONS OF THEORETICAL DISTRIBUTIONS AND PARTITION COEFFICIENTS FOR COUNTER-CURRENT EXTRACTIONS

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The recent paper of Williamson and Craig (1) should advance the use of counter-current extraction processes as a potent laboratory implement. The general usefulness of the method in research laboratories is due to the work of Martin and Synge (2), who showed that the process could be expressed mathematically as a binomial expansion, and to the development of the method and a compact apparatus by Craig (3).

In applications in these laboratories it became necessary to plot a number of theoretical distribution curves. To lighten a tedious calculation task, a method was developed which lent itself to the use of an ordinary adding machine, permitting the calculation of a complete distribution in a very short time. The determination of the partition coefficient from the peak of an experimental distribution curve was also developed as a corollary. The method and calculated values for a series of partition coefficients for both eight and twenty-four transfer processes are presented, since the availability of the data and the ease of calculating new data as needed should make laboratory scale counter-current extraction more generally useful.

The general term of the expansion of the binomial

$$(1) \quad \left( \frac{1}{1+l} + \frac{l}{1+l} \right)^n$$

is

$$(2) \quad T_r = \frac{n!}{r!(n-r)!} \frac{l^r}{(1+l)^n}$$

where  $T_r$  is the fraction present in the  $r$ th tube (plate) of the total material distributed through  $n$  tubes, and  $l$  is the distribution coefficient.

On dividing  $T_r$  by  $T_{r-1}$

$$(3) \quad \frac{T_r}{T_{r-1}} = \frac{n!}{r!(n-r)!} \frac{l^r}{(1+l)^n} \frac{(r-1)!(n+1-r)!}{n!} \frac{(1+l)^n}{l^{r-1}}$$

$$(3, a) \quad T_r = \frac{T_{r-1} l(n+1-r)}{r}$$

$$(3, b) \quad \log T_r = \log T_{r-1} + \log l + \log \frac{n+1-r}{r}$$

The last term of equation (3, b) becomes 24/1, 23/2, 22/3, 3/22, 2/23, 1/24 for  $n = 24$  and 8/1, 7/2, 2/7, 1/8 for  $n = 8$ . The corresponding logarithms are given in Table I.

TABLE I  
Logarithms of  $(n + 1 - r)/r$  for  $n = 8$  and  $n = 24$

$\frac{n+1-r}{r}$	Log	$\frac{n+1-r}{r}$	Log	$\frac{n+1-r}{r}$	Log	$\frac{n+1-r}{r}$	Log
8/1	0 9031	24/1	1 3802	16/9	0 2499	8/17	9 6726 - 10
7/2	0 5441	23/2	1 0607	15/10	0 1761	7/18	9 5898 - 10
6/3	0 3010	22/3	0 8653	14/11	0 1047	6/19	9 4994 - 10
5/4	0 0969	21/4	0 7202	13/12	0 0348	5/20	9 3979 - 10
4/5	9 9031 - 10	20/5	0 6021	12/13	9 9652 - 10	4/21	9 2798 - 10
3/6	9 6990 - 10	19/6	0 5006	11/14	9 8953 - 10	3/22	9 1347 - 10
2/7	9 4559 - 10	18/7	0 4102	10/15	9 8239 - 10	2/23	8 9393 - 10
1/8	9 0969 - 10	17/8	0 3274	9/16	9 7501 - 10	1/24	8 6198 - 10

TABLE II  
Theoretical Per Cent Distributions for Eight Transfer Counter-Current Extractions

$r$	$k$							
	07	08	09	10	11	12	15	20
0	1 4	0 9	0 6	0 4	0 3	0 2	0 1	0 0
1	8 0	5 8	4 2	3 1	2 3	1 8	0 8	0 2
2	19 7	16 3	13 3	10 9	9 0	7 4	4 1	1 7
3	27 6	26 0	24 0	21 9	19 7	17 7	12 4	6 8
4	23 6	26 0	27 0	27 5	27 1	26 5	23 2	17 1
5	13 2	16 6	19 4	21 9	23 9	25 4	27 9	27 3
6	4 6	6 7	8 8	10 9	13 1	15 2	20 9	27 3
7	0 9	1 5	2 2	3 1	4 1	5 2	9 0	15 6
8	0 1	0 2	0 3	0 4	0 6	0 8	1 7	3 9
Total	99 1	100 0	99 8	100 1	100 1	100 2	100 1	99 9

The log of the zero term is obtained by substituting  $r = 0$  in equation (2) and expressing logarithmically

$$(4) \quad \log T_0 = -n \log (1 + k)$$

$$(4, a) \quad \log T_0 = -24 \log (1 + k)$$

$$(4, b) \quad \log T_0 = -8 \log (1 + k)$$

The calculations may now be carried out with the aid of an adding machine. The log of the zero term from equation (4, a) or (4, b) is placed on the tape,  $\log k$  is added,  $\log (n + 1 - r)/r$  is added, and the subtotal re-

corded on the tape is  $\log T_1$ . Successive subtotals obtained by adding  $\log k$  and the corresponding  $\log (n + 1 - r)/r$  are successive  $\log T_r$  values. Calculated values for various  $k$  values for eight transfers and for twenty-four transfers are given in Tables II and III. The numerical fractions obtained from the calculations have been multiplied by 100 per cent and are recorded in Tables II and III as percentages which are more generally

TABLE III

*Theoretical Per Cent Distributions for Twenty-Four Transfer Counter-Current Extractions*

r	k								
	0.7	0.75	0.8	0.9	1.0	1.1	1.2	1.5	2.0
0									
1									
2	0.0	0.0	0.0						
3	0.2	0.1	0.1	0.0	0.0				
4	0.8	0.5	0.3	0.1	0.1	0.0	0.0		
5	2.1	1.5	1.0	0.5	0.2	0.1	0.1		
6	4.7	3.5	2.6	1.5	0.8	0.4	0.2	0.0	
7	8.4	6.8	5.4	3.4	2.1	1.2	0.7	0.2	0.0
8	12.5	10.8	9.2	6.5	4.4	2.9	1.9	0.5	0.1
9	15.5	14.4	13.1	10.3	7.8	5.7	4.1	1.4	0.2
10	16.3	16.2	15.7	14.0	11.7	9.4	7.3	3.2	0.7
11	14.5	15.5	16.0	16.0	14.9	13.2	11.2	6.1	1.8
12	11.0	12.5	13.9	15.6	16.1	15.7	14.6	9.9	3.9
13	7.1	8.7	10.0	12.9	14.9	15.9	16.2	13.7	7.2
14	3.9	5.1	6.4	9.2	11.7	13.8	15.2	16.1	11.4
15	1.8	2.6	3.4	5.5	7.8	10.1	12.2	16.1	15.2
16	0.7	1.1	1.5	3.4	4.4	6.2	8.2	13.6	17.1
17	0.2	0.3	0.6	1.4	2.1	3.2	4.6	9.6	16.1
18	0.0	0.1	0.2	0.5	0.8	1.4	2.2	5.6	12.5
19		0.0	0.0	0.1	0.2	0.5	0.8	2.6	7.9
20				0.0	0.1	0.1	0.2	1.0	3.9
21					0.0	0.0	0.1	0.3	1.5
22							0.0	0.1	0.4
23								0.0	0.1
24									0.0

useful, since experimental samples are rarely of unit weight, and the determination of the distribution may be in units other than weight, *e.g.* activity.

In using Tables II and III, or plots constructed from them, it must be remembered that in the Craig apparatus the upper phase moves, and these calculated values correspond to a moving upper phase. In using separatory funnels, the lower phase moves, and to compare experimental distribution



curves to theoretical curves it is not necessary to recalculate theoretical distributions by means of the reciprocal of the partition coefficient, since merely plotting experimental distributions against tube numbers in descending rather than ascending order has the same effect. Experimental distributions must be expressed as percentages of the whole before plotting the curve for comparison with theoretical curves. An additional condition is inherent in the development, which assumes equal volumes of upper and lower phases.

An experimental partition coefficient may be determined from a sharp peak of an experimental distribution curve. In equation (2),  $T_r$  is a function of  $k$  and the differential of  $T_r$  with respect to  $r$  is the slope of the distribution curve and is zero on passing through the maximum. The operation is simplified by first writing the Napierian logarithm of equation (2), since the maxima of the numerical and the logarithmic curve both fall at the same  $r$ .

$$(5) \quad \ln T_r = \ln n! + r \ln k - \ln r! - \ln (n-r)! - n \ln (1+k)$$

By differentiating equation (5) with respect to  $r$

$$(6) \quad \frac{d \ln T_r}{dr} = \ln k - \frac{d \ln r!}{dr} - \frac{d \ln (n-r)!}{dr}$$

equating the slope to zero

$$(7) \quad \ln k = \frac{d \ln r!}{dr} + \frac{d \ln (n-r)!}{dr}$$

$$(8) \quad \frac{d \ln r!}{dr} = \frac{1}{r!} \frac{d}{dr} [r(r-1)(r-2)(r-3) \dots]$$

$$(8, a) \quad \frac{d \ln r!}{dr} = \frac{1}{r} + \frac{1}{r-1} + \frac{1}{r-2} + \frac{1}{r-3} \dots$$

$$(9) \quad \frac{d \ln (n-r)!}{dr} = \frac{1}{(n-r)!} \frac{d}{dr} [(n-r)(n-1-r)(n-2-r)(n-3-r) \dots]$$

$$(9, a) \quad \frac{d \ln (n-r)!}{dr} = - \left[ \frac{1}{n-r} + \frac{1}{n-1-r} + \frac{1}{n-2-r} + \frac{1}{n-3-r} \dots \right]$$

substituting in equation (7) from equations (8, a) and (9, a), and letting  $n = 24$

$$(10) \quad \ln k = \left[ \frac{1}{r} + \frac{1}{r-1} + \frac{1}{r-2} + \dots \right] - \left[ \frac{1}{24-r} + \frac{1}{23-r} + \frac{1}{22-r} + \dots \right]$$

Equation (10) which is used for calculating the Napierian logarithm of a partition coefficient giving a peak at any tube number, is fortuitously

adaptable to the use of an adding machine. The calculations were performed for  $n = 24$  to correspond to the Craig apparatus, and the results are given in Table IV. Every third value corresponds to one in an eight transfer series, and each value corresponds to a value for an even numbered  $r$  in a forty-eight transfer series. Since interpolation for higher  $r$  values is inaccurate, the values for the odd numbered  $r$  may be obtained from a curve based on Table IV.

The per cent purity of a sample is properly given by the ratio of the area under the experimental curve, corrected by adjustment for the partition

TABLE IV  
*Partition Coefficient Corresponding to Peak of Experimental Curve*

$n$			$k$	$n$			$k$	$n$			$k$
8	24	48		8	24	48		8	24	48	
0	0	0	0.023	3	9	18	0.614	6	17	34	2.33
	1	2	0.065		10	20	0.725		18	36	2.84
	2	4	0.101		11	22	0.852		19	38	3.53
1	3	6	0.163	4	12	24	1.0	7	20	40	4.61
	4	8	0.217		13	26	1.17		21	42	6.12
	5	10	0.283		14	28	1.38		22	44	8.91
2	6	12	0.352	5	15	30	1.63	8	23	46	15.3
	7	14	0.430		16	32	1.94		24	48	43.2
	8	16	0.515								

coefficient corresponding to the peak, to the area under the theoretical curve for the same partition coefficient. The area can be shown to be

$$(11) \quad A = \frac{(n!)^n (k)^{n^2/2}}{(1+k)^n}$$

which need not be used, since the area is the product of  $T_r$  and a function of  $r$  which is the same for both the experimental and the theoretical curves. The ratio of the areas, and hence the percentage, may be expressed by the ratio of the experimental  $T_r$  to the corresponding theoretical  $T_r$ . The  $T_r$  selected for the ratio should be of the tube containing the minimum impurity and can be selected by inspection of the curves, but is not necessarily the maximum  $T_r$ .

#### SUMMARY

A simplified method for calculating theoretical distributions for counter-current extractions with either separatory funnels or the Craig apparatus has been presented, together with a series of calculated values. A quick

determination of the partition coefficient from the location of the peak of an experimental curve has also been pointed out

Thanks are due Mr Martin Kuna for valuable assistance rendered in numerous discussions and by the critical application of the theoretical curves

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## BIOLOGICAL PRECURSORS OF URIC ACID\*

### I THE RÔLE OF LACTATE, ACETATE, AND FORMATE IN THE SYNTHESIS OF THE UREIDE GROUPS OF URIC ACID

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Search for the biological sources of the ureide carbon atoms of uric acid has centered chiefly around compounds similar in structure to the ureide group of uric acid. One of the earliest studies on the precursors of uric acid was made by Wiener (3), who postulated that uric acid was formed by the condensation of 2 moles of urea with 1 of tartronic acid. Although this hypothesis was at first attractive, it has, however, been disproved as the mechanism of the biosynthesis of uric acid by several investigators using both *in vivo* and *in vitro* techniques (4-10).

Pyrimidines (11), which are likewise related to purines, have also been excluded as precursors of uric acid, since they are oxidized almost quantitatively to urea by the mammal. More recent isotopic experiments of Plentl and Schoenheimer (12) also provide convincing evidence that pyrimidines do not participate in uric acid synthesis.

A more serious difference of opinion, however, has persisted among investigators until recently concerning histidine and arginine as the precursors of purines or purine derivatives. The opening investigations on this subject were made by Ackroyd and Hopkins (13), who postulated that the imidazole nucleus of allantoin could be derived from either of these amino acids. Subsequent data obtained by comparing uric acid or allantoin excretion of animals fed diets high and low in these amino acids have not been altogether conclusive or in agreement (14-19). Results obtained by the isotope tracer technique, however, do not confirm the point of view that histidine or arginine may be precursors of uric acid (10). The feeding of  $N^{15}$ -labeled ammonium citrate to rats and pigeons led to the incorporation of  $N^{15}$  into purines of the tissues and uric acid or allantoin of the excreta in much greater concentrations than in histidine or arginine of the tissue proteins. Moreover, uric acid isolated after the administration to the pigeon of arginine labeled with  $N^{15}$  did not contain significant amounts of  $N^{15}$  (20). These findings have led to the conclusion that the

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reactions proposed by Krebs and Henseleit (21) for the synthesis of urea in the rat do not apply to the synthesis of a structurally related substance, the ureide group of uric acid

The demonstration by Baines and Schoenheimer (10) that  $N^{15}$  of ammonium citrate is readily incorporated into uric acid has suggested that the ureide carbon might be derived from some simple metabolic unit. In order to test this possibility several isotopic compounds important in intermediary metabolism, namely  $C^{13}O_2$ ,  $HC^{13}OOH$ ,  $CH_3C^{13}OOH$ ,  $NH_2CH_2C^{13}OOH$ ,  $CH_3CHOHC^{13}OOH$ , and  $C^{13}H_3C^{13}HOHCOOH$ , were prepared and administered to pigeons. The excreted uric acid was degraded by chemical procedures and information has been gained concerning the origin of each of the carbon atoms of uric acid. In the present paper experiments dealing with the sources of the ureide carbon will be reported. In the following paper experiments concerning the precursors of the carbon chain of uric acid are described.

#### EXPERIMENTAL

*Organic Syntheses*—Isotopic compounds were synthesized for the most part by procedures described by Sakami, Evans, and Guin (22). These procedures consisted of standard organic reactions modified for synthesis of compounds with  $C^{13}$ . Carboxyl-labeled acetate was prepared from isotopic  $CO_2$  and methyl iodide by the Guignard reaction, carboxyl-tagged glycine by the hydrolysis of ethyl phthalimidoacetate (23) prepared from potassium phthalimide and carboxyl isotopic ethyl bromoacetate. This latter compound was synthesized according to the method of Auwers and Bernhardt (24). Carboxyl-labeled lactate was synthesized according to the method of Ciarnier and Kistiakowsky (25),  $\alpha$ - or  $\beta$ -tagged lactate by the method of Ciarnier and Kistiakowsky (25) as modified by Sakami, Evans, and Guin (22). Formic acid was prepared by the hydrolysis of hydrogen cyanide (26).

#### Procedure

Pigeons used in this experiment weighed 295 to 323 gm with an average weight of 310 gm. All birds had been fasted for 1 day. During the experiment the pigeon was kept on a wire screen in a large desiccator through which was passed a stream of  $CO_2$ -free air. Collections of respiratory  $CO_2$  were made at 15, 30, 60, or 120 minute intervals during the experiment by passing the respiratory gases through a solution of NaOH and by precipitating the carbonate as  $BaCO_3$ .

3 cc of a solution containing 20 mg of  $N^{15}H_4Cl$  (3.7 atoms per cent excess) and from 0.5 to 1.0 mm of the isotopic compound were alternately fed or injected intraperitoneally at intervals of 2 hours. Acidic substances

were administered as the sodium salt. Glycine and the two types of isotopic lactate were administered at the rate of 0.5 mM per hour,  $\text{NaHCO}_3$  and formate at the rate of 0.75 mM per hour, and acetate at the rate of 1 mM per hour. The method of administration of solutions was changed in the experiment with  $\text{NaHC}^{13}\text{O}_3$ . In this case 2.5 cc of a solution containing 0.38 mM of  $\text{NaHC}^{13}\text{O}_3$ , 10 mg of  $\text{N}^{15}\text{H}_4\text{Cl}$ , and 25 mg of glucose were administered every 30 minutes. The duration of the experiments was from 16 to 20 hours, except in the experiments with glycine and formate. Because of the small amount of glycine available to us, four administrations were made over a period of 8 hours and the experiment was not concluded until the end of the 10th hour. The experiment with formate was concluded at the end of the 12th hour. In the experiments with acetate, formate, and lactate the pigeons were sacrificed and the contents of the peritoneal cavity and enteric tract collected. These washings were cleared with colloidal iron and copper-lime and the unabsorbed volatile acids or lactate were determined by the methods of Friedemann (27) and Friedemann and Graeser (28) respectively. From these determinations the amounts of these substances absorbed during the experimental period were calculated. 93, 94, 78, and 91 per cent of the administered acetate, formate, carboxyl-labeled lactate, and  $\alpha, \beta$ -tagged lactate respectively were absorbed.

In a supplementary experiment performed with a rat, 3 cc of a solution containing 1 mM of carboxyl-labeled acetate, 50 mg of glucose, and 30 mg of  $\text{NH}_4\text{Cl}$  were fed every hour for a period of 4 hours. At the conclusion of the experiment urea carbon was liberated from the urine as  $\text{CO}_2$  by urease. Samples of respiratory  $\text{CO}_2$  were also collected.

In the experiments with pigeons uric acid was isolated from the excreta and purified by the method of St. John and Johnson (29) as modified by Fisher (30). Uric acid obtained by this procedure was suspended in 40 cc of water, dissolved with a minimum amount of  $\text{NaOH}$ , decolorized with carboraffin, and reprecipitated with  $\text{HCl}$ . This procedure was repeated until a pure white product was obtained.

*Degradation of Uric Acid*—The conventional numbering system, shown in Scheme 1 of the following paper, has been used to designate the position of the carbon atoms of the uric acid molecule. Carbons 2 and 8, representing the uride carbons of the pyrimidine and imidazole rings of uric acid respectively, may be separated from the other carbons of uric acid and from each other by appropriate degradation procedures. The first procedure is a modification of a method described by Edson and Krebs (31). The exact details of the method of degradation of uric acid with alkaline  $\text{MnO}_2$  into  $\text{CO}_2$ , glyoxylic acid, and urea may be found in the accompanying paper. Urea produced by the alkaline oxidation of uric acid was extracted

by a suitable procedure and converted into  $\text{CO}_2$  by urease (32). Since this urea is derived equally from carbons 2 and 8, isotopic analysis of this fraction gives an average of the  $\text{C}^{13}$  concentration of these 2 carbon atoms. When this urea contained no excess of isotope, it was concluded that neither carbon 2 nor 8 was isotopic and further degradation procedures were usually not employed. If, however, this fraction contained  $\text{C}^{13}$ , a second oxidation procedure was used by which it was possible to obtain carbons 2 and 8 separately (33). In this procedure urea containing carbon 8 was obtained by oxidizing uric acid with  $\text{KClO}_3$  in strong  $\text{HCl}$  (34). Alloxan, the other product of the reaction, was reduced to alloxantin with  $\text{H}_2\text{S}$  (34) and the insoluble crystalline alloxantin was isolated. Alloxantin was subsequently oxidized by  $\text{PbO}_2$  to urea containing carbon 2 (34). The oxidation with  $\text{PbO}_2$ , a modification of a procedure described by Liebig and Wohler, was carried out at  $100^\circ$  for 20 minutes. The urea fractions obtained in each case were treated with urease to liberate  $\text{CO}_2$ .

### Results

*Oxidation of Administered Substances*—No attempt was made in these studies to follow in detailed manner the rate of oxidation of the various substances administered to pigeons. In previous studies on rats it has been found that 2 hours after the administration of carboxyl-labeled acetate (35), carboxyl-labeled lactate (36),  $\alpha$ - and  $\beta$ -labeled lactate (37), and carboxyl-labeled glycine (38) respectively, 36, 16, 8, and 4 per cent of the administered isotope are liberated as  $\text{CO}_2$  in the respiratory gases. Since in the present experiments measurement was not made of the total amount of  $\text{C}^{13}$  excreted, it is not possible to express  $\text{C}^{13}$  excreted in terms of per cent of the amount administered. However, an approximate estimation of the rate of oxidation of the administered substance may be made by comparing their "coefficients of oxidation." The "coefficient of oxidation" is arbitrarily defined as

$$100 \times \frac{\text{C}^{13} \text{ concentration of } \text{CO}_2 \text{ of respiratory gases}}{\text{mm excess C}^{13} \text{ given per hr}}$$

where millimoles of excess  $\text{C}^{13}$  given per hour  $\times 100 = \text{C}^{13}$  concentration (atoms per cent excess) of carbon atoms labeled  $\times$  the number of atoms labeled  $\times$  the rate of administration of isotopic compound.

As may be seen in Column 8 of Table I, the carboxyl carbon atoms of acetate and glycine and the  $\alpha$ - and  $\beta$ -carbon atoms of lactate are all excreted as  $\text{CO}_2$  by the pigeon to approximately the same extent. There is the possibility that the carboxyl carbon atom of lactate is excreted at a rate somewhat greater but in the same order of magnitude as the labeled carbon atoms of the other compounds.

Since the metabolism of isotopic formate has not been studied previously *in vivo*, a more thorough investigation was made of the rate of oxidation of this substance. In Table II is reported the  $C^{13}$  concentration of the

TABLE I  
*Precursors of Ureide Carbon Atoms of Uric Acid in Pigeon*

Experi ment No	Precursor	Rate given	<sup>13</sup> C concentration (atoms per cent excess)				Oxida tion coeffi- cient
			Labeled carbon	Uric acid car bon No		Respir atory CO	
				2	8		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
		<i>mm per hr</i>					
1†	C*O <sub>2</sub>	0 75	8 13	0 02	0 02	0 28	
2†	HC*OOH	0 75	3 34	2 41	2 41	0 01	0 5
3	CH <sub>2</sub> C*OOH	1 00	5 82	2 02	2 10	0 26	4 5
4	NH <sub>2</sub> CH <sub>2</sub> C*OOH	0 50	5 20	0 00	0 00	0 12	4 6
5†	<i>dl</i> -CH <sub>2</sub> CHOHC*OOH	0 50	8 80	0 01	0 01	0 25	5 8
6	<i>dl</i> -C*H <sub>2</sub> C*HOHCOOH	0 50	5 40	0 10	0 10	0 11	4 1

$C^*$  = carbon atom labeled with  $C^{13}$

† The values reported for the  $C^{13}$  concentrations of uric acid carbons 2 and 8 in Experiments 1, 2, and 5 are the  $C^{13}$  concentrations of the mixed urea carbons. Further degradation procedures to separate carbons 2 and 8 were not performed in these experiments.

TABLE II  
*Appearance of Isotopic  $CO_2$  in Respiratory Gas after Administration of Isotopic Formate to Pigeon*

3 cc. of a solution containing 8.7 mm of isotopic formate (3.34 atoms per cent excess  $C^{13}$ ), 0.75 gm. of glucose, and 6.3 mm of  $NH_4Cl$  in a total volume of 18 cc. were administered six times at 2 hour intervals to a 360 gm. pigeon.

Hrs after 1st administration	$C^{13}$ concentration of respiratory $CO_2$ (atoms per cent excess)	Hrs after 1st administration	$C^{13}$ concentration of respiratory $CO_2$ (atoms per cent excess)
1	0.01	6	0.02
2	0.02	7	0.00
3	0.03	8	0.02
4	0.01	9	0.01
5	0.02	10	0.02
Average			0.013

respiratory  $CO_2$  of samples taken every hour for the first 10 hours of the 12 hour experiment. As may be seen, the  $C^{13}$  concentration of the  $CO_2$  is very low throughout the entire experiment and within the experimental



error of the mass spectrometer. When the average of the excess  $C^{13}$  found in these samples is used in the calculation of the "oxidation coefficient" (Table I), a value for the "oxidation coefficient" is obtained which is far below that of the other compounds. It would thus seem that under the conditions of this experiment formate is not further oxidized or, at most, very slowly oxidized to  $CO_2$  by the pigeon. The absence of isotopic  $CO_2$  in the respiratory gases was not due to lack of absorption of the isotopic formate. The inability of the pigeon to oxidize formate, however, does not mean that formate may not enter into metabolic reactions. As shown in Table I, formate participates in the synthesis of uric acid.

*Metabolic Origin of Carbons 2 and 8 of Uric Acid*—After the administration to pigeons of the six compounds listed in Table I, it was found that the carboxyl carbon atoms of acetate and formate and the  $\alpha$ - or  $\beta$ -carbon atom of lactate contributed the ureide carbon atoms in the synthesis of uric acid, but that  $CO_2$  and the carboxyl carbon atoms of lactate and glycine did not participate to any measurable extent. In two instances where  $C^{13}$  appeared in ureide carbon of uric acid after the administration of isotopic compounds (*i.e.* Experiments 3 and 6), uric acid was degraded by the procedure which permitted the separation of carbon atoms 2 and 8 for isotopic analysis. The fact that the concentrations of  $C^{13}$  of carbon atoms 2 and 8 in both experiments are essentially equal argues for the belief that both ureide carbon atoms are formed from the same source (*i.e.*, acetate, lactate, or formate) and that a part of the uric acid molecule is not derived preferentially from a pyrimidine or imidazole ring compound of the body tissues or diet.

From the concentration of  $C^{13}$  in the carbon of the substances administered and in carbon atoms 2 and 8 of the uric acid isolated and from other data reported in the literature, an estimation may be made of the extent to which the above compounds participate in uric acid synthesis. After the administration of formate containing 3.34 atoms per cent  $C^{13}$  to a pigeon, uric acid was isolated containing 2.41 atoms per cent excess  $C^{13}$  in the ureide carbon atoms. It may thus be calculated that 72 per cent of the ureide carbon of the newly formed uric acid was derived from the administered formate. In addition to formate which is readily utilized in the synthesis of ureide, the carboxyl carbon of acetate is also an important precursor of this group, an indication that formate and acetate may be closely linked in avian metabolism. After the administration of acetic acid labeled in the carboxyl group with 5.82 atoms per cent excess  $C^{13}$ , it was found that the uric acid isolated contained 2.02 and 2.10 atoms per cent excess  $C^{13}$  in the 2 and 8 positions respectively. This demonstrates that the  $(2.10/5.82) \times 100$  or approximately 35 per cent of the ureide carbon of uric acid was derived from the acetate administered.

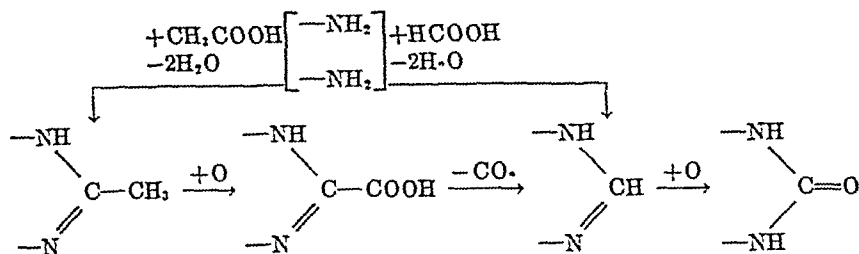
From the experiments of Bloch and Rittenberg (39) it is known that a 300 gm rat may form 50 mm of acetate per day. Although the difference of experimental conditions makes it difficult to estimate from these data the amount of acetate formed by a 300 gm pigeon, a rough approximation may be made to ascertain the order of magnitude. Since Experiment 3 was of 16 hour duration, the pigeon may have formed as much as 33 mm of acetate. Since 16 mm of isotopic acetate were administered during this time, 1 part of isotopic acetate may have been diluted by 2 parts of metabolically produced non-isotopic acetate. The  $C^{13}$  concentration of the carboxyl carbon of acetate which takes part in metabolic reactions would be approximately one-third that of the carboxyl carbon of the administered acetate or 1.91 atoms per cent excess. This latter value approximates very closely the value of the  $C^{13}$  concentration of the ureide carbon atoms of uric acid. These calculations may indicate that acetate is the mother substance from which ureide carbon is derived and suggest that formate is either an intermediate between acetate and the ureide group or may readily be converted into an intermediate of this reaction.

Isotopic carbon of  $\alpha, \beta$ -labeled lactate is probably converted into ureide carbon by virtue of the fact that the carboxyl carbon of acetate may be derived from the  $\alpha$ -carbon of lactate. The utilization of the  $\alpha$ -carbon of lactate for ureide synthesis is perhaps less than was expected. It should be noted that the experiments with  $\alpha, \beta$ -tagged lactate and ureide formation have only qualitative importance, in view of the fact that in this one instance the administered lactate was contaminated with non-isotopic formate which may have served to dilute isotopic ureide carbon formed by the metabolism of  $\alpha, \beta$ -tagged lactate by the bird. It was not appreciated at the time of the experiment with  $\alpha, \beta$ -tagged lactate that formate played a rôle in avian metabolism. In a subsequent experiment with carboxyl-tagged lactate, contaminating formate was removed from solution by an appropriate procedure.

*Administration of Carboxyl-Labeled Acetate to Rat*—In an experiment supplementary to those with pigeons, acetate labeled in the carboxyl carbon with 5.20 atoms per cent excess  $C^{13}$  was fed to a rat at the rate of 1 mm per hour for 4 hours. Urea was collected at the mid-point of the experiment. It was found that the  $C^{13}$  concentration of urea carbon was 0.27 atom per cent excess as compared to 0.50 atom per cent excess for the respiratory  $CO_2$ . Since  $CO_2$  is a source of urea carbon both *in vivo* (40) and *in vitro* (21, 41, 42), it was concluded that the carboxyl carbon of acetate does not directly supply urea carbon in the rat. This is in contrast to the finding that the carboxyl carbon of acetate is a major source of the ureide carbon of uric acid in the pigeon.

## DISCUSSION

Data presented in the present paper have served to confirm the belief of recent investigators that arginine and histidine are not direct sources of the ureide carbon of uric acid. To the already accepted fact that urea cannot be incorporated into uric acid has been added the information that the ureide group of uric acid in the pigeon and urea carbon in the rat derive their carbon from dissimilar sources. In addition, it has been demonstrated that formate may enter into avian metabolism and that the carboxyl carbon of acetate may be utilized in the biosynthesis of a group containing but 1 carbon atom. Although little is known about the formation of imidazole or pyrimidine rings in the living organism, there is a chemical precedent for the formation of imidazole ring structures by the condensation of diamino compounds with the carboxyl groups of fatty acids. Thus benzimidazole may be formed by the condensation of *o*-phenylenediamine and formic acid (43). The participation of both acetic and formic acids in uric acid formation in the bird indicates that a similar mechanism is involved in the biosynthesis of not only the imidazole but also the pyrimidine ring. The utilization of formate and the non-utilization of  $\text{CO}_2$  suggest that a reduced form of uric acid (*i.e.*, a purine) is an intermediate in uric acid synthesis. Östrom, Östrom, and Krebs (44) have shown that hypoxanthine may be formed in pigeon liver slices from simple metabolic units and have suggested that hypoxanthine is an intermediate of uric acid formation by the intact organism. Our data are in complete accord with this postulation. The hypothesis that the biosynthesis of the ureide groups of purines involves a condensation of a diamino compound and a fatty acid is supported by the recent work of Stetten and Fox, Shive and Roberts, and Shive *et al*. Stetten and Fox (45) have isolated in pure form an amine which accumulates in cultures of various bacteria as a result of the addition of sulfonamides. Shive and Roberts (46) have demonstrated that purines are capable of overcoming partially the bacteriostatic action of sulfonamides in certain instances and have postulated that the sulfonamides compete with a factor involved in the conversion of a purine precursor into the purine itself. This hypothesis has been recently borne out by Shive *et al* (47), who have characterized the amine isolated by Stetten and Fox as 5(4)-amino-4(5)-imidazolecarboxamide. In view of the work reported from various laboratories, the utilization of fatty acid,  $\text{CO}_2$ ,  $\text{NH}_3$ , and glycine (1) in the synthesis of 5(4)-amino-4(5)-imidazolecarboxamide and the further condensation of this compound with a second molecule of fatty acid to form hypoxanthine might be predictable steps in uric acid synthesis. In order to reconcile the utilization of both formate and acetate in uric acid synthesis, the following series of reactions are tentatively proposed to explain the derivation of ureide carbon from acetate.



In these reactions it is postulated that the carboxyl group of acetate is first linked to nitrogen atoms with the formation of a methyl-substituted amidine structure. Upon further oxidation, a carboxyl group might be formed from the methyl carbon. Decarboxylation would yield the ureide carbons attached to hydrogen atoms as they are in the hypoxanthine molecule. This same structure might conceivably be formed directly by the condensation of the carboxyl group of formate with 2 atoms of nitrogen. It is evident that the above reactions are only hypothetical and require further investigation to establish their validity.

If formate enters into uric acid synthesis by virtue of its metabolic relationship to an intermediate in the reactions involved in the conversion of acetate carbon to ureide carbon, it may be concluded that this new oxidative pathway of acetate metabolism does not constitute a second mechanism for the *complete* oxidation of acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . This conclusion is based on the observation that the carbon of isotopic formate does not appear in the respiratory gases to a significant degree after its administration.

From the data presented in Table I, additional information concerning formate metabolism may be obtained. The fact that formate is an important source of the ureide carbon of uric acid and that  $\text{CO}_2$  does not enter into this reaction to a measurable extent demonstrates that  $\text{CO}_2$  may not be reduced to formate in the bird as it is in certain bacterial preparations (48). Furthermore the inability of the carboxyl carbon of lactate to serve as a source of ureide carbon demonstrates that formate is not a significant metabolic product of the carboxyl carbon of lactate or pyruvate in the bird as it is in the metabolism of some bacteria (49, 50).

#### SUMMARY

1. Six compounds labeled with  $\text{C}^{13}$  (*i.e.*  $\text{C}^{13}\text{O}_2$ ,  $\text{HC}^{13}\text{OOH}$ ,  $\text{CH}_3\text{C}^{13}\text{OOH}$ ,  $\text{NH}_2\text{CH}_2\text{C}^{13}\text{OOH}$ ,  $\text{CH}_3\text{CHOHC}^{13}\text{OOH}$ , and  $\text{C}^{13}\text{H}_5\text{C}^{13}\text{HOHCOOH}$ ) have been prepared and administered to fasted pigeons. The excreted uric acid was isolated, purified, and degraded by procedures which permitted the isolation of both the ureide carbon atoms for isotopic analysis.

2 The carboxyl carbon of acetate and formate and the  $\alpha$ - (or  $\beta$ -) carbon of lactate may participate in ureide synthesis, but the labeled carbon atoms of the other compounds studied do not. It is believed that acetate is the biological source of the ureide carbon and that formate is either an intermediate or may readily be converted into an intermediate of this reaction. It is further believed that the isotopic carbon of  $\alpha, \beta$ -labeled lactate is converted into ureide carbon by virtue of the fact that the carboxyl carbon of acetate may be derived metabolically from the  $\alpha$ -carbon of lactate.

3 The present experiments demonstrate that formate may be utilized in avian metabolism, even though it is not oxidized to an appreciable extent.

4 The utilization of acetate in ureide formation, although a new oxidative path of acetate metabolism, probably does not constitute a second pathway for the *complete* oxidation of acetate to  $\text{CO}_2$  and  $\text{H}_2\text{O}$ .

5  $\text{CO}_2$  and the carboxyl groups of lactate or pyruvate are not precursors of formate in the pigeon as they are in certain bacterial preparations.

6 In a supplementary experiment it was found that the carboxyl group of acetate is not a direct precursor of urea in the rat. These experiments demonstrate that the carbon of urea and ureide carbon of uric acid have different metabolic origins.

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## BIOLOGICAL PRECURSORS OF URIC ACID\*

### II THE RÔLE OF LACTATE, GLYCINE, AND CARBON DIOXIDE AS PRECURSORS OF THE CARBON CHAIN AND NITROGEN ATOM 7 OF URIC ACID

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Proteins, fats, and carbohydrates or their metabolic derivatives have been investigated in great detail as possible precursors of the carbon chain of uric acid (3-11). The usual procedure has been to test the effect of the administration of these substances on the excretion of uric acid under a variety of experimental conditions. For the most part, experiments have been performed on the fasted animal. The administration of proteins and to a lesser extent of carbohydrates has been shown to increase the excretion of uric acid. Fats, however, either had no effect or caused a decrease in uric acid output. The amino acids, alanine, glycine, asparagine, and glutamine have been shown to cause increased excretion of uric acid in man under conditions in which ammonia or urea did not (12-15). Deaminated derivatives of some of these amino acids have been tested for their effect on uric acid output. Fisher (16) has claimed that L-lactate may increase uric acid excretion in the bird. In an attempt to demonstrate an effect of organic acids in the excretion of uric acid in man, Gibson and Doisy (17) found that lactate and glycolic acid decreased excretion, whereas alanine and pyruvate greatly increased it.

Experiments *in vivo* wherein ingested substances were compared for their ability to increase uric acid excretion above the normal level of the fasted subject are open to criticism on the basis of the work of Lennox (18). Lennox has shown that the fasted subject gradually increases his stores of blood and tissue uric acid which may be released by the ingestion of certain foodstuffs, notably carbohydrates and proteins or amino acids. The increased excretion of uric acid, caused by the administration of a substance, may not then represent participation of the administered substance in the synthesis of uric acid but rather a depletion of the body stores.

The use of *in vitro* techniques has likewise brought no definite answer

\* Preliminary reports of these studies have been published (1, 2). Aided by a grant from the American Cancer Society.



to the question of the precursors of the carbon chain of uric acid. The suggestion of Wiener that tartaric acid is involved in uric acid synthesis has been disproved by several investigators (19, 20). A great many carbon-containing substances other than tartaric acid have been tested for their ability to stimulate uric acid production in tissue slices. Schuler and Reindel (19) have shown that liver extract is capable of forming an unknown precursor which in the presence of liver and kidney slices and ammonium chloride is converted to uric acid. Östrom, Östrom, and Kiebs (21) have been able to demonstrate that asparagine, glutamine, pyruvate, and oxalacetate may stimulate the formation of hypoxanthine in pigeon liver slices. As pointed out by Kiebs and his coworkers, the stimulatory effects of these acids do not indicate that their carbon chains necessarily participate directly in purine synthesis. It is known, for example, that energy-producing oxidations must accompany many biological syntheses.

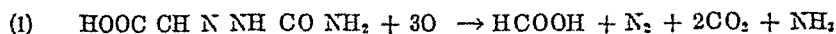
For the most part, the experimental procedures used to demonstrate whether a given substance participates in uric acid synthesis depend upon the ability of this substance to increase uric acid excretion *in vivo* or its formation *in vitro*. The biosynthesis of uric acid is undoubtedly a complicated procedure involving many reactions, one of which may be the limiting one. If a tested substance is not the limiting factor of the synthesis, it may participate in purine formation without increasing materially the rate of uric acid synthesis. This inherent difficulty in determining the precursors of a synthetic reaction is overcome with the use of isotopically marked compounds. In the present paper, several intermediates of protein, fat, and carbohydrate metabolism have been labeled with  $C^{13}$  and administered to pigeons. The excreted uric acid was degraded by procedures which permitted the separation of each of the carbon atoms of the carbon chain of uric acid for isotopic analysis. This use of compounds labeled with  $C^{13}$  has permitted the identification of some of the carbon precursors of uric acid. Additional information concerning the nitrogen precursors of uric acid has been obtained by administering ammonium chloride containing  $N^{15}$  simultaneously with each of these organic compounds.

#### EXPERIMENTAL

The general procedure used in the synthesis of isotopic compounds and the manner of their administration to pigeons have been described in an accompanying communication (22). In order to separate for isotopic analysis carbon atoms 4, 5, and 6 of the carbon chain of uric acid excreted after the administration of isotopic  $CO_2$ , formate, acetate, glycine, or lactate, an aliquot of uric acid was oxidized with alkaline  $MnO_2$  to hydroxy-

acetylene diureide carboxylic acid, which was subsequently decomposed by acid into allantoin and  $\text{CO}_2$  (23). This  $\text{CO}_2$  is derived from carbon 6 of uric acid. Allantoin was then hydrolyzed in alkaline solution to allantoic acid, which upon subsequent acid hydrolysis decomposed into urea and glyoxylic acid (24).

Glyoxylic acid was isolated as the semicarbazone. This derivative was then oxidized by  $\text{KMnO}_4$  by the following equations



Since the first reaction occurs much more rapidly than the second, a sample of  $\text{CO}_2$  collected during the first 7 minutes of reaction contains mainly carboxyl carbon of glyoxylic acid plus the semicarbazide carbon. A sample of  $\text{CO}_2$  collected during the next 4 hours resulting from the oxidation of  $\text{HCOOH}$  contains the aldehyde carbon of glyoxylic acid.

Fischer and Ach (25) have reported a series of experiments in which methylated purines were oxidized in alkaline solution to allantoin. The chief product of oxidation of 1- or 7-methyluric acid is  $\beta$ -methylallantoin (the methylated nitrogen being adjacent to the carbonyl group of the carbon chain). Likewise, the chief product isolated after alkaline oxidation of 3- or 9-methyluric acid was  $\alpha$ -methylallantoin. From the work of Fischer and Ach it would then appear that the carbonyl carbon of the 2-carbon chain of allantoin, which upon hydrolysis becomes the carboxyl carbon of glyoxylic acid, is derived from carbon 5 of uric acid (see Scheme 1, Reaction III). The aldehyde carbon of glyoxylic acid is, by the same argument, derived from carbon 4.

Although the formation of glyoxylic acid by the mechanism described in part by Fischer and Ach (25), by Behrend (26), and by Siemonsen (27) (*i.e.* Reaction III, Scheme 1) is thought to be the main pathway of uric acid oxidation by alkaline  $\text{MnO}_2$ , there is the possibility that side reactions may occur. Evidence obtained from our own experiments, however, supports the conclusion of Fischer and Ach and others that uric acid is degraded to allantoin (and hence to glyoxylic acid) as described in Reaction III and also, further, that side reactions do not play an important rôle in this degradation.

In Experiment 5, Table I, with carboxyl-labeled lactate, the  $\text{CO}_2$  liberated upon acidification of the alkaline  $\text{MnO}_2$  solution contained 0.25 atom per cent excess  $\text{C}^{13}$ , whereas the carboxyl carbon of glyoxylic acid contained no excess of isotope. If uroxyanic acid had been a major intermediate in the degradation of uric acid (Reaction I, Scheme 1), then these 2 carbon atoms should have contained  $\text{C}^{13}$  in approximately equal concentrations. The complete absence of isotope in the carboxyl carbon of glyoxylic



resulted in glyoxylic acid in which the carboxyl and aldehyde carbon atoms are derived from carbon atoms 4 and 5 of uric acid respectively. In a reverse manner degradation of uric acid via Reaction III would have resulted in glyoxylic acid in which the carboxyl and aldehyde carbon atoms are derived from carbon atoms 5 and 4 of uric acid respectively. If the degradation of uric acid had taken place by means of Reactions II and III in equal proportions, then the glyoxylic acid should have contained  $C^{13}$  in the carboxyl and aldehyde groups in equal concentrations. Since one of the carbon atoms, namely that in the carboxyl group of glyoxylic acid, contained no isotope, whereas the other contained 0.37 atom per cent excess  $C^{13}$ , it may be concluded, in view of the work of Fischer and Ach with N-methylated uric acids, that uric acid is degraded predominantly by

TABLE I  
*Biological Precursors of Carbon Chain of Uric Acid*

Experiment No	Precursor	$C^{13}$ concentration (atoms per cent excess)						
		Labeled carbons	Respiratory $CO_2$	Uric acid carbon No				
				6	4 + 5	5	4	4 (corrected for $CO_2$ )
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
1	$C^*O_2$	8.13	0.28	0.25	0.04	0.00†	0.07†	
2	$HC^*OOH$	3.34	0.01	0.01	0.08			
3	$CH_3C^*OOH$	5.82	0.26	0.22	0.04	0.00	0.07	0.00
4	$NH_2CH_2C^*OOH$	5.20	0.12	0.11		0.14	1.16	1.13
5	<i>dl</i> $CH_3CHOHC^*OOH$	8.80	0.25	0.26		0.00	0.37	0.31
6	<i>dl</i> $C^*H_2C^*HOHCOOH$	5.40	0.11	0.09		0.14	0.07	0.04

$C^*$  = carbon atoms labeled with  $C^{13}$

† Calculated on the basis of the procedure indicated in the text

way of Reaction III and that Reaction II does not play a significant rôle in the procedure.

The presence of a relatively small amount of isotopic carbon in the carboxyl group of glyoxylic acid in Experiment 4, Table I, with carboxyl-labeled glycine might leave open the possibility that a small part of uric acid is degraded by one or both of the two side reactions described (*ie* Reaction I or II). There is, however, another equally plausible explanation for the presence of isotope in carbon 5 of Experiment 4, Table I. Reference to the procedure for the oxidation of glyoxylic acid semicarbazone will show that  $CO_2$  (containing carbon 5) produced during the first 7 minutes of reaction with acid permanganate might be contaminated by the secondary oxidation of formic acid (containing carbon 4). If this

should be the case, then 12.8<sup>1</sup> per cent of formic acid may be degraded in the first 7 minutes. Such an oxidation in the other experiments would not have yielded a high enough concentration of isotope in carbon 5 to be detected.

*Experimental Details of Degradation Procedure*—The procedure of Edson and Krebs (24) was used with certain modifications. 80 mg. of uric acid were dissolved in 10 cc. of 0.1 N NaOH and oxidized by 10 cc. of a MnO<sub>2</sub> suspension for 30 minutes at 38°. After filtration, the MnO<sub>2</sub>-free solution was transferred to an aeration apparatus and 0.7 cc. of 10 N HNO<sub>3</sub> was added. The solution was aerated rapidly for 10 minutes at room temperature to remove any extraneous CO<sub>2</sub>, the temperature was then raised to 38° and carbon 6, as CO<sub>2</sub>, was slowly evolved and collected during the next 60 minutes. The solution was then transferred to a 50 cc. centrifuge tube and neutralized to pH 7. Enough Na<sub>2</sub>CO<sub>3</sub> was added to make a 0.1 M solution (350 mg.) and the tube placed in a boiling water bath for 20 minutes for the hydrolysis of allantoin to allantoic acid. The tube was removed from the bath, its contents made distinctly acid to thymol blue with 10 N HNO<sub>3</sub>, and then returned to the boiling water bath for an additional 4 minutes for the hydrolysis of allantoic acid to glyoxylic acid and urea. Nitric acid was used because lead is employed later in the procedure.

The solution was cooled, again neutralized to pH 7, and 5 cc. of basic lead acetate were added to precipitate the glyoxylic acid (28). After centrifuging, the supernatant was saved for future extraction of urea, while the precipitate was transferred to a 15 cc. centrifuge tube with 10 to 13 cc. of water. To this precipitate were added 1.5 cc. of 4 N H<sub>2</sub>SO<sub>4</sub>. The suspension was stirred well, centrifuged, and the supernatant containing glyoxylic acid was poured through a filter into another 15 cc. centrifuge tube. The precipitate was washed with 0.4 cc. of H<sub>2</sub>O and the washings

<sup>1</sup> To calculate the possible contamination of carbon 5 by carbon 4 in the degradation of glyoxylic acid semicarbazone with acid permanganate in the glycine experiment, let  $n$  = millimoles of the total formic acid decomposed,  $y$  = millimoles of glyoxylic acid semicarbazone decomposed,  $x = n/y$  = the fraction of formic acid decomposed, and  $c$  = isotope concentration of the mixture of CO<sub>2</sub> removed during the first 7 minutes of reaction = 0.07 atom per cent excess C<sup>13</sup>. The assumptions are made that the carboxyl and aldehyde carbons of glyoxylic acid contain respectively 0.00 and 1.16 atoms per cent excess C<sup>13</sup>. Therefore

$$(3) \quad c = \frac{n(1.16) + 2y(0)}{n + 2y}$$

Dividing the numerator and the denominator of the right hand side of Equation 3 by  $y$  and substituting  $x = n/y$

$$(4) \quad c = \frac{x(1.16)}{x + 2}$$

$$(5) \quad x = \frac{2c}{1.16 - c} = \frac{0.14}{1.09} = 0.128 \text{ or } 12.8\%$$

combined with the first supernatant. To this glyoxylic acid solution were added 250 to 300 mg of semicarbazide hydrochloride, and the glyoxylic semicarbazone was allowed to crystallize overnight in a cold room. The semicarbazone derivative was washed with small portions of water, was treated with carboraffin, and recrystallized from 2 to 4 cc of water. After standing for 24 hours in the cold room, the semicarbazone was centrifuged and dried *in vacuo* for 4 hours. The yields varied from 13 to 26 mg. It was frequently necessary to do a second degradation to obtain a quantity of glyoxylic acid semicarbazone sufficient for the acid permanganate oxidation to follow. Melting points were taken with unknown, mixed, and known samples. The purity of the known specimen was determined by a Veibel titration (29) and also by an acidimetric titration. Mol wt known, 130, found, Veibel, 134, acidimetric 132, m p 204–205°.

To separate the 2 carbon atoms of glyoxylic acid, 1.5 cc of 1.5 N  $\text{KMnO}_4$  and 4 cc of 1 N  $\text{H}_2\text{SO}_4$  were added to a 30 cc solution containing 20 mg of glyoxylic acid semicarbazone. The oxidation was carried out at 38°. The  $\text{CO}_2$  evolved during the first 7 minutes was collected in one sample and the  $\text{CO}_2$  evolved during the next 7 minutes discarded. The  $\text{CO}_2$  collected after the first 14 minutes results from the slow oxidation of formic acid and represents carbon 4 of uric acid. The conclusions concerning the mechanism of oxidation of glyoxylic acid semicarbazone (reactions (1) and (2), above) are drawn by analogy from the mechanism of oxidation of  $\alpha$ -ketoglutaric acid semicarbazone by acid permanganate (30) and the known fact that formic acid is slowly oxidized under these conditions (31). In separate experiments it was possible to show that 2 of the 3 carbon atoms of glyoxylic acid semicarbazone are liberated rapidly during the first 10 to 12 minutes of reaction and that the 3rd carbon is liberated much more slowly thereafter. Since the  $\text{CO}_2$  evolved during the first 7 minutes contained the carboxyl carbon of glyoxylic acid plus the semicarbazide carbon, the  $\text{C}^{13}$  concentration of carbon 5 of uric acid was obtained by multiplying the  $\text{C}^{13}$  concentration of this  $\text{CO}_2$  fraction by 2.

The supernatant saved after the precipitation of glyoxylic acid with basic lead acetate was evaporated to dryness and urea extracted with absolute alcohol. The alcohol was evaporated and the urea redissolved in water. The aqueous solution was acidified, aerated with  $\text{H}_2\text{S}$  to precipitate any lead still present, and filtered. Urea was decomposed to ammonia and  $\text{CO}_2$  by urease. The results of experiments in which an investigation was made of the precursors of the ureide carbons of uric acid are reported in the accompanying paper. In three experiments, the two ureide groups of uric acid containing nitrogen atoms 1 plus 3 and 7 plus 9 respectively were obtained separate from each other in the form of urea by a procedure previously described (22). These samples of urea were con-

verted by urease into ammonia for eventual determination of their  $N^{15}$  concentrations in the mass spectrometer

### Results

The results of six experiments are included in Table I. The administration of the six isotopic compounds listed has made possible the identification of some precursors of the carbon chain of uric acid as well as nitrogen 7 of the ureide group.

*Metabolic Origin of Carbon 6 of Uric Acid*—Of the six isotopic compounds administered to pigeons, only one,  $CO_2$ , was directly involved as a precursor of carbon 6 of uric acid. After the administration of isotopic bicarbonate containing 8.13 atoms per cent  $C^{13}$ , uric acid was isolated from the excreta containing 0.25 atom per cent excess  $C^{13}$  in carbon 6. During the 14th hour of a 16 hour experiment, five samples of respiratory  $CO_2$  were collected at 15 minute intervals. The isotopic analyses for these successive samples were 0.32, 0.27, 0.27, 0.27, and 0.30 atom per cent excess  $C^{13}$ , thus demonstrating that under the conditions of these experiments  $C^{13}$  was expired at a fairly constant rate. It should be noted that a  $C^{13}$  concentration of carbon 6 of uric acid (0.25) parallels closely the  $C^{13}$  concentration of the respiratory  $CO_2$  (average value 0.28), indicating that carbon 6 of uric acid is probably derived directly from  $CO_2$ . This was borne out in subsequent experiments where isotopically marked organic compounds were administered. As is shown in Columns 5 and 4, Table I, the concentrations of  $C^{13}$  in carbon 6 and in the respiratory  $CO_2$  closely parallel each other in all five of the experiments. Thus, the appearance of  $C^{13}$  in position 6 in these experiments probably results from the incorporation of  $C^{13}O_2$  produced by the metabolism of these isotopic organic compounds and does not represent the direct utilization of labeled carbon atoms of organic compounds as precursors of carbon 6 of uric acid. Special mention should be made of formate in this connection. The demonstration by Utter, Werkman, and Lipmann (32) and by Lipmann and Tuttle (33) that formic acid may be formed from  $CO_2$  by certain bacterial extracts and that formate and acetyl phosphate condense to form pyruvate has increased the interest in formate as a possible intermediate of  $CO_2$  assimilation reactions. The experiment with formate reported in Table I demonstrates, however, that formate does not participate as an intermediate during the incorporation of  $CO_2$  into uric acid. After the administration of isotopic formate, carbon 6 of uric acid isolated from the excreta contained no excess of  $C^{13}$ .

*Metabolic Origin of Carbons 4 and 5*—All of the compounds administered led to the enrichment of the 2 carbon fragment of uric acid containing carbons 4 and 5. After the administration of  $NaHCO_3$  (8.13 atoms per cent excess  $C^{13}$ ) and acetate (containing 5.82 atoms per cent excess  $C^{13}$  in the

carboxyl carbon), the glyoxylic acid derived from the degradation of the resulting uric acid contained in both instances 0.04 atom per cent excess  $C^{13}$ , while the respiratory  $CO_2$  contained 0.28 and 0.26 atom per cent excess  $C^{13}$  respectively. By degradation of the glyoxylic acid semicarbazone of the acetate experiment it was found that all of the isotope of this compound was located in the aldehyde carbon or what had been carbon 4 of uric acid ( $\pm 0.07$  atom per cent excess). Not enough glyoxylic acid semicarbazone was available in the experiment with isotopic sodium bicarbonate to permit further degradation. Since, however, the administration of either isotopic bicarbonate or isotopic acetate resulted in the enrichment of the glyoxylic acid and respiratory  $CO_2$  to about the same extent ( $\pm 0.04$  and 0.27 atom per cent excess, respectively), it seems permissible to assume that the appearance of  $C^{13}$  in carbons 4 plus 5 is due solely to assimilation of  $CO_2$  in both experiments. Furthermore, it is assumed that the assimilation of  $CO_2$  into the 2 carbon fraction of uric acid representing carbons 4 and 5 takes place in position 4 and that in Experiment 1, Table I, the concentration of isotope in this position is 0.07 atom per cent excess  $C^{13}$ , the same as in Experiment 3. In subsequent experiments correction was made for the assimilation of  $CO_2$  in position 4 of uric acid by the following equation

(6) Concentration of  $C^{13}$  in carbon 4 due to assimilation of  $C^{13}O_2$

$$= \frac{0.07}{0.28} \times \text{concentration of } C^{13} \text{ in respiratory } CO_2$$

After the administration of carboxyl-labeled glycine, lactate, and  $\alpha, \beta$ -labeled lactate containing  $C^{13}$  in concentrations indicated in Table I, 1.16, 0.37, and 0.07 atom per cent excess  $C^{13}$  were found in carbon 4 of uric acid respectively (Column 8, Table I). When these values are corrected for the assimilation of  $CO_2$ , they are respectively 1.13, 0.31, and 0.04 atom per cent excess  $C^{13}$ . These values are recorded in Column 9, Table I. It is thus seen that the carboxyl groups of lactate and glycine are both sources of carbon 4 of uric acid. The low concentration of  $C^{13}$  in carbon 4 of uric acid after the administration of  $\alpha, \beta$ -tagged lactate indicates that these carbon atoms are not significant precursors of carbon 4. The  $\alpha$ - or  $\beta$ -carbon atoms of lactate are, however, significant sources of carbon 5 of uric acid. After the administration of lactate containing 5.40 atoms per cent excess  $C^{13}$  in the  $\alpha$ - and  $\beta$ -carbon atoms, it was found that carbon 5 of uric acid contained 0.14 atom per cent excess  $C^{13}$ . The administration of the other isotopic compounds did not lead to enrichment of this carbon atom to a significant extent. The appearance of  $C^{13}$  in carbon 5 of uric acid after the administration of carboxyl-labeled glycine is probably due to a contamination of carbon 5 by carbon 4 during the degradation procedure, and



hence has no metabolic significance. Such a contamination of carbon 5 by carbon 4 becomes apparent only when the concentration of  $C^{13}$  of carbon 4 is relatively large, as it is in Experiment 4.

The administration of isotopic formate resulted in a definite enrichment of the glyoxylic acid derived from the degradation of uric acid. It is unfortunate that sufficient material was not available for a further degradation of this compound. In view of the fact that formate is not converted to  $C^{13}O_2$  to a significant extent, it is probable that the isotope present in carbons 4 plus 5 represents a real utilization of formate carbon.

In Table II an estimation has been made of the extent to which lactate and glycine carbon atoms enter positions 4 and 5 in the synthesis of uric acid. In Columns 3 and 4 a calculation is made of the extent to which

TABLE II  
*Comparison of Utilization of Carbon Atoms of Lactate and Glycine in Synthesis of Carbon Chain of Uric Acid*

Experiment No	Precursor	Per cent indicated carbon of uric acid derived from labeled carbon of precursor		Specific coefficient of utilization of racemic acids	Specific coefficient of utilization, of L acids
		Carbon atom 4	Carbon atom 5		
(1)	(2)	(3)	(4)	(5)	(6)
4	$NH_2CH_2C^*OOH$	22.0		44.0	44.0
5	$DL-CH_2CHOHC^*OOH$	3.5		7.0	14.0
6	$DL-C^*H_3C^*HOHCOOH$		2.6	5.2	10.4

$C^*$  = carbon atoms labeled with  $C^{13}$

† In Experiments 4, 5, and 6 the isotopic substances were administered at the rate of 0.5 mm per hour to fasted pigeons weighing 295, 323, and 323 gm respectively. All weights were so close to 300 gm that correction for pigeon weight was not made in the above calculations.

carbon atoms 4 and 5 of uric acid were derived directly from the administered isotopic carbon of each of the three compounds listed. The percentage value is obtained by dividing the  $C^{13}$  concentrations of carbon 4 or 5 (Columns 9 and 7, Table I, respectively) by the  $C^{13}$  concentration of the isotopic carbon of the administered substance (Column 3, Table I) and multiplying this ratio by 100. For purposes of further discussion this value will be called  $A$ . Perhaps a more accurate comparison of the utilization of lactate and glycine carbon in uric acid formation may be made by calculating the specific coefficient of utilization of these compounds, as have Bloch and Rittenberg (34) in their studies of acetate formation in the rat. For purposes of comparison "the specific coefficient of utilization" of glycine and lactate carbon in uric acid synthesis is defined as  $A/M$  where  $M$  repre-

sents the number of millimoles of substance administered to a 300 gm pigeon per hour. This definition is similar to but not identical with the term "specific coefficient of utilization" as used by Bloch and Rittenberg. According to Bloch and Rittenberg the compound with the highest coefficient of utilization is the most immediate precursor of a given product of reaction. In Column 5, Table II, are recorded the coefficients of utilization of the two isotopic species of lactate and of carboxyl-labeled glycine, assuming that the D and L forms of lactate are equally well utilized in uric acid synthesis. Since the data of Fisher (16) suggest that D-lactate is not utilized for uric acid synthesis in the pigeon, the coefficients of utilization have been recalculated (Column 6, Table II), assuming that only the L-lactate participates in this reaction. From the coefficients of utilization of these compounds it may be seen that the carboxyl carbon of lactate is incorporated into the 4th position of uric acid to about the same extent that the  $\alpha$ -carbon<sup>2</sup> of lactate is incorporated into position 5. Furthermore, the carboxyl carbon of glycine is incorporated into position 4 to a much greater extent than is the carboxyl carbon of lactate. These calculations would indicate, therefore, that glycine is the most immediate source of carbon 4 and that lactate is converted into glycine prior to its utilization for uric acid synthesis. Since the carboxyl and  $\alpha$ -carbon atoms of lactate are utilized in the synthesis of uric acid in positions 4 and 5 respectively, it seems valid to assume that the  $\alpha$ -carbon of glycine is a major source of carbon 5 of uric acid. This assumption is borne out by further experiments with  $N^{15}$  which are reported below.

*Metabolic Origin of Nitrogen 7*—In Experiments 3, 4, and 6, Table I, uric acid was degraded in such a manner that nitrogen atoms 1 and 3 were obtained together, but separate from nitrogen atoms 7 and 9. In Table III is recorded the  $N^{15}$  concentration of these nitrogen atoms in experiments in which  $N^{15}H_4Cl$  (3.70 atoms per cent excess  $N^{15}$ ) was administered with either carboxyl-labeled acetate, carboxyl-labeled glycine, or  $\alpha,\beta$ -labeled lactate. Non-isotopic glucose was administered routinely in all experiments. It may be seen that, after the administration of either carboxyl-labeled acetate or  $\alpha,\beta$ -tagged lactate together with  $N^{15}H_4Cl$ , the  $N^{15}$  concentration of nitrogen atoms 1 plus 3 was equal to that of nitrogen atoms 7 plus 9. After the administration of  $N^{15}H_4Cl$  and glycine containing isotopic carbon but non-isotopic nitrogen, the  $N^{15}$  concentration of nitrogen atoms 1 plus 3 was 0.712, as compared to 0.548 in nitrogen atoms 7 plus 9. The lower concentration of  $N^{15}$  in nitrogen atoms 7 plus 9 indicates that the non-isotopic nitrogen of glycine participates directly in the synthesis of the

<sup>2</sup> The assumption is made that the  $\alpha$ - rather than the  $\beta$  carbon of lactate is utilized in the formation of carbon 5, since the carboxyl and  $\alpha$  carbons are adjacent to each other.

imidazole ring of uric acid. Since the carboxyl carbon of glycine is the precursor of carbon atom 4 of uric acid, it is concluded that glycine nitrogen is the source of nitrogen atom 7 and that the  $\alpha$ -carbon of glycine is the source of carbon atom 5.

Moreover, these experiments with  $N^{15}$  are further evidence that glycine, rather than lactate, is the immediate source of carbons 4 and 5. They also demonstrate that there is a rapid synthesis of glycine by the organism during uric acid synthesis. The equality of  $N^{15}$  concentration in nitrogen atoms 1 plus 3 and 7 plus 9 after the administration of lactate or acetate indicates that little or no preformed glycine is available to the organism for uric acid synthesis but that most of it is readily synthesized from carbohydrate precursors, possibly lactate, and  $N^{15}H_4Cl$ .

Shemin and Rittenberg (35) have recently reported experiments in which they fed  $N^{15}$ -labeled glycine to a human and measured the  $N^{15}$  concentra-

TABLE III

*$N^{15}$  Concentration of Nitrogen Atoms of Uric Acid after Administration of  $N^{15}H_4Cl$  together with Carbon Isotopic Compounds*

Experiment No	$C^{13}$ precursor	$N^{15}$ concentration (atoms per cent excess) uric acid nitrogen atom No	
		1 + 3	7 + 9
3	$CH_3C^*OOH$	0.856	0.853
4	$NH_2CH_2C^*OOH$	0.712†	0.548
6	$C^*H_3C^*HOHCOOH$	0.606	0.593

$C^*$  = carbon atoms labeled with  $C^{13}$

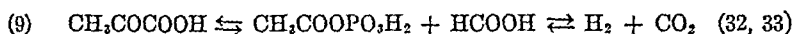
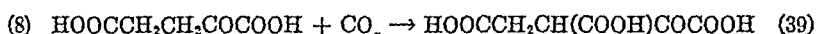
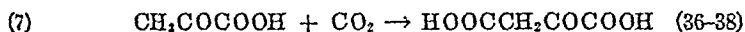
† Since this sample was lost, this value was calculated from the  $N^{15}$  concentration of the total uric acid molecule and the  $N^{15}$  concentration of nitrogen atoms 7 plus 9.

tions of the individual nitrogen atoms of the uric acid excreted at various intervals after the administration of the isotopic compound. They have found that the nitrogen of glycine is the source of nitrogen 7 of uric acid. These experiments not only confirm the conclusion of the present authors (1) that glycine is a precursor of uric acid, but demonstrate that the reactions of uric acid synthesis in the pigeon and human are probably very similar. The fact that uric acid is the major product of nitrogen metabolism in the bird but not in the human had previously led to speculation that the reactions of uric acid synthesis in these two organisms were fundamentally different.

#### DISCUSSION

Although it was primarily the purpose of this work to ascertain the sources of uric acid carbon, the data presented in this and the accompanying paper provide limited information on reactions involved in its synthesis.

*Incorporation of CO<sub>2</sub> into Uric Acid*—With the finding that CO<sub>2</sub> is incorporated into 2 positions of the carbon chain of uric acid, it is of interest to determine, if possible, whether these CO<sub>2</sub> assimilation reactions proceed via known reactions or whether new CO<sub>2</sub> reactions may be involved. At present three reactions are known by which CO<sub>2</sub> may be assimilated into carbon to carbon linkage by animal and bacterial tissues. All of these reactions involve pyruvate (or lactate) or metabolic derivatives of this substance. They are as follows:



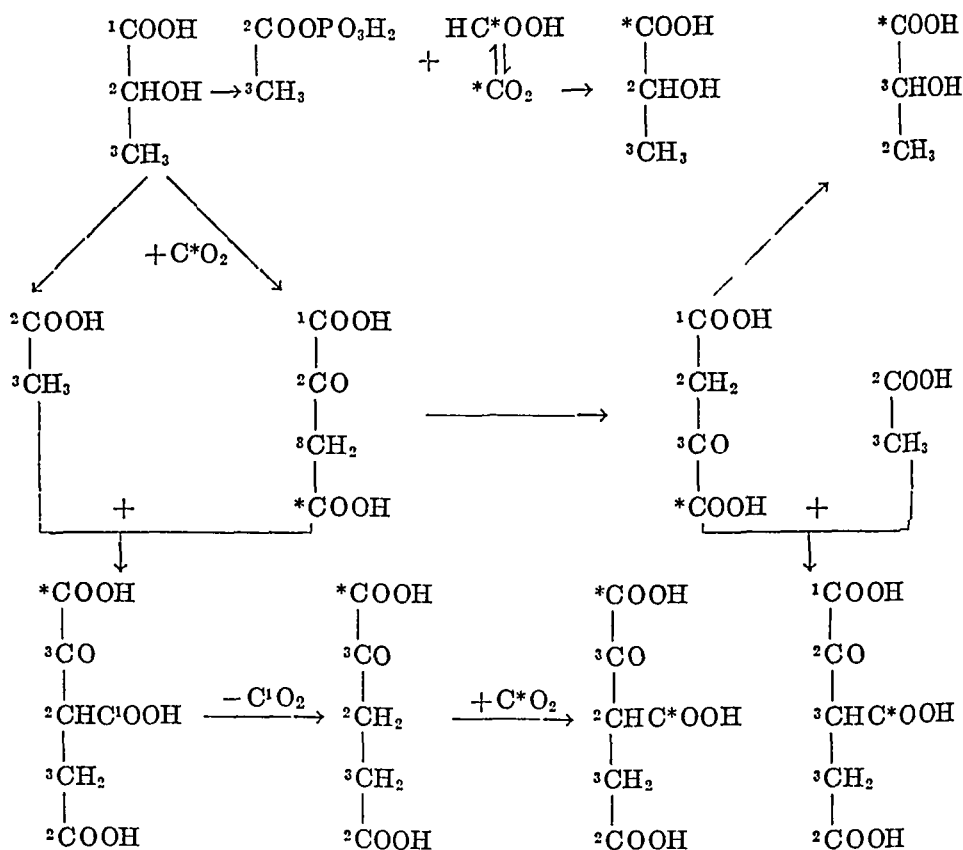
The assimilation of CO<sub>2</sub> into position 4 of uric acid was readily explainable on the basis of Equation 7 after it was found that the carboxyl carbon of lactate was likewise a source of this carbon atom of uric acid. From the experiments of Wood *et al.* (37) and Evans *et al.* (40), it is known that carboxyl-tagged lactate is readily formed when non-isotopic pyruvate is incubated with pigeon liver minces or extracts in the presence of isotopic bicarbonate. This takes place by the addition of CO<sub>2</sub> to pyruvate, forming oxalacetate. Isotope is then distributed equally between the two carboxyl groups of oxalacetate, which may then be decarboxylated to carboxyl isotopic pyruvate. Reduction of this latter compound gives carboxyl isotopic lactate. It is thus apparent that the carboxyl carbon of both lactate and CO<sub>2</sub> might appear in the same position (*i.e.* 4) of uric acid. The assimilation of CO<sub>2</sub> into position 6 of uric acid is not readily explained on the basis of the above three reactions, however. From the foregoing discussion (see "Results") the conclusion was drawn that carbon atoms 6, 5, and 4 of the 3-carbon chain of uric acid may be derived from CO<sub>2</sub>, the  $\alpha$ -carbon atom of lactate, and the carboxyl carbon atom of lactate respectively.

It is important to study the possibility that the 3-carbon chain of uric acid may be formed by an established assimilation reaction of CO<sub>2</sub> with lactic acid. In Scheme 2 are summarized the known reactions of lactic acid which involve the fixation of CO<sub>2</sub>. The carboxyl,  $\alpha$ -, and  $\beta$ -carbons of lactate as well as CO<sub>2</sub> are traced as they form the carbon atoms of known metabolic products. In no instance is the arrangement of carbon atoms in either lactate or a metabolic derivative of lactate such that a 3-carbon chain may be split out wherein the carbons are derived from CO<sub>2</sub>, the  $\alpha$ -carbon of lactate, and the carboxyl carbon of lactate in the order named. This would indicate that the 3-carbon chain of uric acid is not derived from any of these metabolic derivatives of lactic acid and that consequently none of

the  $\text{CO}_2$  assimilation reactions involved in these paths of metabolism of lactate is involved in the synthesis of uric acid. The authors believe, therefore, that the incorporation of  $\text{CO}_2$  into carbon 6 of uric acid takes place by a  $\text{CO}_2$  assimilation reaction hitherto undescribed.<sup>3</sup>

### SCHEME 2

*Tracing of Carbon Atoms of Lactate and  $\text{CO}_2$  during Formation of Metabolic Derivatives of Lactate by Known  $\text{CO}_2$  Assimilation Reactions*



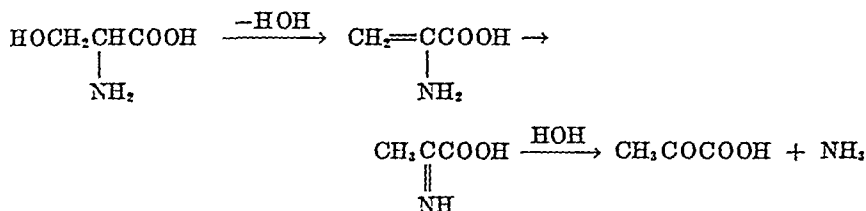
The carboxyl,  $\alpha$ -, and  $\beta$ -carbons of lactate and  $\text{CO}_2$  are designated by the symbols 1, 2, 3, and \* respectively

*Possible Relationship of Lactate to Uric Acid Synthesis*—Although none of the reactions included in Scheme 2 satisfactorily explains the presence of  $\text{C}^{13}$  in the indicated positions of the uric acid molecule after the administra-

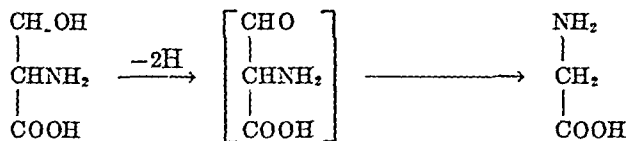
<sup>3</sup> The recent observation by Barker and Elsdon (41) that  $\text{CO}_2$  is assimilated into acetate and glycine during the fermentation of uric acid by *Clostridium cylindro sporum* demonstrates that  $\text{CO}$  assimilation is also involved in the breakdown as well as the synthesis of uric acid. It seems at present, however, that the  $\text{CO}_2$  reactions of the fermentation of uric acid by *Clostridium cylindrosporum* are not related to those involved in its synthesis by the pigeon

tion of isotopic bicarbonate or either of the two types of isotopically labeled lactate, certain reactions have recently been proposed for the metabolism of lactate and related derivatives which could account for some of the observed facts. These reactions involve the conversion of lactate to glycine, a 3- to a 2-carbon unit. These reactions are as follows

(10) Conversion of serine to pyruvate (Chargaff and Sprinson (42))



(11) Conversion of serine to glycine (Shemin (43))



Shemin (43) has shown that serine may be converted to glycine in the rat by oxidation of the  $\beta$ -carbon atom and it seems likely that the same mechanism for the formation of glycine from serine is present in avian metabolism. Chargaff and Sprinson (42) have demonstrated the conversion of serine to pyruvate by *Bacterium coli* and Binkley (44) has reported that a similar reaction may take place in animal tissues. Chargaff and Sprinson have proposed the reactions of Equation 10 as a possible mechanism for the conversion of serine to pyruvate. If these reactions are reversible, they would provide a pathway for the conversion of lactate to glycine when taken in conjunction with the experiments of Shemin. If the above series of reactions do explain the conversion of lactate to glycine, it may be readily seen that the carboxyl and  $\alpha$ -carbon atoms of lactate should constitute the carboxyl and  $\alpha$ -carbon atoms of glycine respectively. This is in accord with the experimental data.

In view of the experiments with  $\text{N}^{15}$  reported in this paper and in that of Shemin and Rittenberg (35), it seems likely that glycine itself is the nucleus around which the other carbon and nitrogen atoms of uric acid become associated during its biosynthesis. The participation of acetate in the formation of the ureide groups of uric acid and the increasing importance of acetylation reactions in intermediary metabolism raise the question of whether the acetylation of glycine might be one of the steps involved in uric acid synthesis. The possible reactions whereby acetate and formate

may enter into the synthesis of the ureide groups of uric acid have been discussed in the accompanying paper

*Acetate and Glycine Not Interconvertible in Pigeon*—Experiments on glycogen formation from acetate and glycine have strongly indicated that these compounds are not metabolized by the same pathways, since glycine is glycogenic (45) whereas acetate is not (46). Our experiments with uric acid confirm this belief. The carboxyl carbon of acetate is a major contributor to positions 2 and 8 of uric acid (22), but does not enter to a significant extent into position 4. In a reverse manner the carboxyl carbon of glycine is a major contributor to position 4 but does not enter into positions 2 and 8 (22). It is thus evident that the 2 carbon atoms of glycine are not directly utilized to form the 2-carbon structure of acetate and *vice versa*. Almquist and Mecchi (47) have reported that acetate may replace glycine in the nutrition of the chick. In unpublished experiments by one of us (J. M. B.) the results of Almquist and Mecchi could not be duplicated. The inclusion of acetate in the diet did not restore the loss of growth produced by the omission of glycine from the basal diet. The experiments with acetate and glycine concerning uric acid synthesis in the pigeon likewise fail to substantiate the findings of Almquist and Mecchi.

Although a good deal has been learned recently about the reactions of acetate oxidation, little is known about reactions leading to glycine oxidation or conversion of glycine to carbohydrate. The data presented in this paper on uric acid synthesis may offer a clue to these reactions. It has been shown that the 3-carbon chain of uric acid is formed by reactions involving  $\text{CO}_2$  and the  $\alpha$ -carbon of glycine. A similar but not necessarily identical reaction may be involved in the oxidative or glycogenic metabolism of glycine. Thus glycine might be converted to lactate by a reversal of the reaction proposed above for the conversion of lactate to glycine. Lactate thus formed could be either oxidized or utilized in the synthesis of glycogen by conventional routes of metabolism of this latter compound.

The authors wish to acknowledge with appreciation the aid of Dr. D. W. Wilson in the preparation of samples for isotopic analysis and of Dr. Samuel Guin in the synthesis of organic compounds. They also wish to thank Mr. James Houck of the Department of Physics, University of Pennsylvania, and Dr. Sidney Weinhouse, Catalytic Development Corporation, Marcus Hook, for the analysis of  $\text{C}^{13}$  samples, and Dr. David Rittenberg of Columbia University for  $\text{N}^{15}$  analyses.

#### SUMMARY

1. By administering organic compounds labeled with  $\text{C}^{13}$  to pigeons and degrading the excreted uric acid, it has been shown that  $\text{CO}_2$  is the

source of carbon 6 of uric acid and that the carboxyl carbon of glycine is the source of carbon 4. By indirect experiments with  $N^{15}H_4Cl$  it was demonstrated that the amino nitrogen of glycine is probably the source of nitrogen 7 of uric acid. The  $\alpha$ -carbon of glycine is probably the source of carbon 5.

2. It is believed that the incorporation of  $CO_2$  into position 6 of uric acid takes place by a  $CO_2$  assimilation reaction hitherto undescribed.

3. The carboxyl and  $\alpha$ -carbons of lactate are also incorporated into positions 4 and 5 respectively of uric acid, but to a lesser extent than is the carboxyl carbon of glycine incorporated into position 4. It is thought, therefore, that lactate is converted to glycine by reactions involving the conversion of serine to glycine.

4. Glycine and acetate are not directly interconvertible in the metabolism of the pigeon. The authors have suggested that the formation of a 3-carbon intermediate of carbohydrate metabolism by a reaction involving  $CO_2$  and the  $\alpha$ -carbon of glycine might be a step in the oxidative and glycolytic metabolism of glycine.

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# CRYSTALLINE ACTIVATED PROTEIN B (CHYMOTRYPSIN B)\*

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The protein isolated from beef pancreas (1) was recently identified as the zymogen of a proteolytic enzyme and provisionally called protein B (2)

In this paper a method of crystallization of the activated enzyme is described Further properties of this enzyme have been studied, suggesting that the activated protein B belongs to the group of chymotrypsins The strongest evidence for this conclusion came from the work of Dr Fruton (3) in which the specificity of the activated protein B toward several synthetic peptides has been investigated The activity was found to be similar to that of the group of chymotrypsins In view of our own, and particularly Dr Fruton's results, we have proposed the name chymotrypsin B for the active enzyme and chymotrypsinogen B for the unactivated form

## EXPERIMENTAL

The unactivated protein B was prepared according to the method previously described for protein B<sub>2</sub> (2) Since it has been shown that forms B<sub>1</sub> and B<sub>2</sub> were interchangeable, it was found convenient to prepare protein B in the form B<sub>2</sub> only Prior to the activation, protein B was recrystallized a minimum of five times

5 gm of the crystalline zymogen were dissolved in 75 ml of 0.2 M borate buffer, pH 7.8, and the pH adjusted to 7.8 1 mg of crystalline trypsin, equivalent to 5.5 Kunitz (4) trypsin units, was added to each 1.5 gm of the zymogen The solution was left at 5° Every 24 hours a minimum of eight 1 ml samples of enzyme containing from 1 to 100 γ of protein was incubated with 1 ml of standard 1 per cent casein (in phosphate buffer, pH 7.6) for 20 minutes at 37° The activity<sup>1</sup> was determined on the trichloroacetic acid centrifugate spectrophotometrically by the method of Kunitz

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† Some of the data included in this report were taken from a thesis to be submitted by Kenneth D Brown to the Graduate School of Marquette University in partial fulfillment of the degree of Master of Science

<sup>1</sup> No numerical unit of activity was introduced For the purpose of this work, it was found most convenient to compare the activity of an unknown with a standard (Fig 4) When necessary, the potency of the unknown was expressed in per cent of the standard

(4) The results of one such experiment are shown in Fig. 1. Maximum activity was usually attained in 72 hours.

At this time the mixture was transferred to a cellophane dialyzing sac and dialyzed for 72 hours against 0.01 M acetate buffer, pH 5.5 at 5°, the outside buffer being changed frequently. A heavy, amorphous precipitate resulted. This precipitate was centrifuged with difficulty (3 hours at

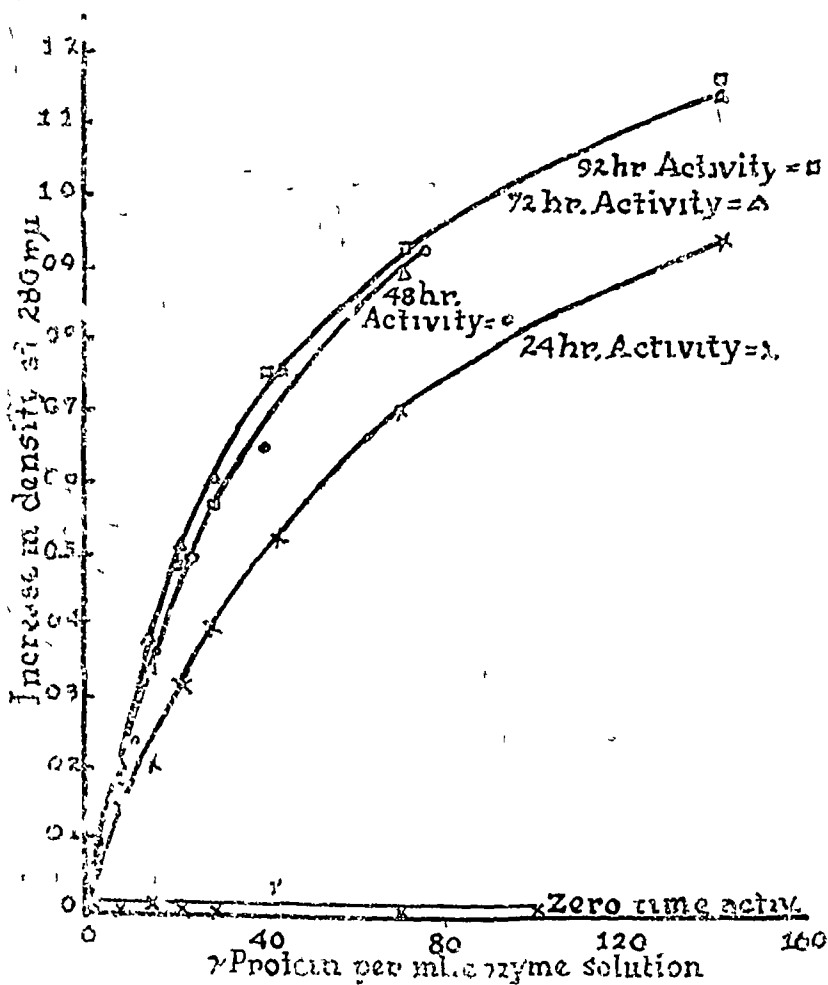
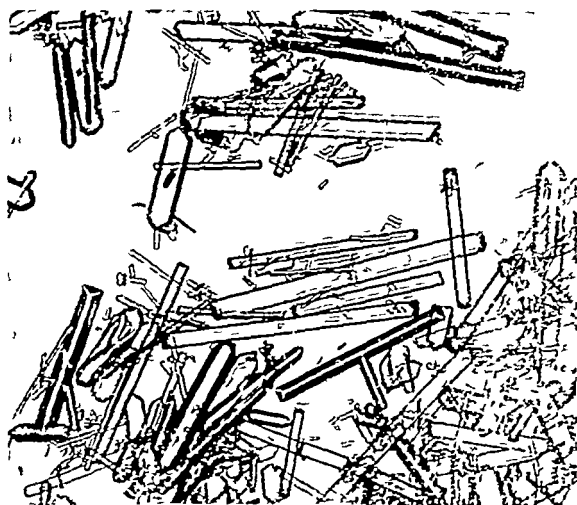


FIG. 1. Activation of protein E by trypsin

5000 R.P.M.) and dissolved in a minimum of water, made pH 4.0 by the addition of N sulfuric acid. The small amount of gelatinous, insoluble material was removed by centrifugation and the clear centrifugate was dialyzed against 0.01 M acetate buffer, pH 5.5. The resulting precipitate was semicrystalline. The precipitate was centrifuged and dissolved at pH 7.8. This solution was dialyzed against 0.01 M acetate buffer, pH 5.5. The

The activity of the enzyme on casein has been compared with that of crystalline chymotrypsin and crystalline trypsin. The results are shown in Fig. 5. The curve obtained with crystalline trypsin was identical with Fig. 14 of Kunitz (4). The difference between activated protein B and trypsin was very pronounced in respect to the initial velocity of the reaction and to the end-point of digestion. This confirmed our previous findings (2) concerning the non-identity of activated protein B and trypsin. By comparing the curves representing activated protein B and crystalline trypsin, it was seen that the shapes of both were very similar. They differed, however, in that the velocity of the reaction with activated protein B was considerably slower.



resulting precipitate was crystalline (Fig. 2). These crystals were dissolved at pH 10 and dialyzed against 0.01 M acetate buffer, pH 5.5. The resulting precipitate was crystalline (Fig. 3). It is of interest to note that, when the crystallization was induced by lowering the pH, the needle form shown in Fig. 2 was obtained. On the other hand, when the crystallization was induced by raising the pH, the prisms (short and elongated, Fig. 3) resulted. It was not uncommon to obtain from both procedures macroscopic crystals. As a standard procedure, the activated protein was recrystallized four times, the methods described above being alternated. At this stage the active enzyme was lyophilized with no significant loss of activity and in this form the



FIG. 2. Crystalline activated protein B obtained by lowering the pH from 7.8 to 5.5.  $\times 210$ .

preparation was stable. Fig. 4 shows the results of an experiment in which three successive crystallizations of the same batch of activated protein B were tested for activity. The other two sets of points in Fig. 4 represent two successive recrystallizations of a different batch of activated protein B. As is seen, all points are within the experimental error of the method and are represented by one curve. The successive activity determinations of the same batch of activated protein B without intervening recrystallizations resulted in a greater variation as is shown in Fig. 4. It was concluded, therefore, that crystalline activated protein B represented an enzyme of uniform activity which persisted upon subsequent recrystallizations.

and 10  $\gamma$  of activated protein B respectively. These three tubes were incubated for the next 20 minutes and the reaction was stopped by the addition of 1 ml. of 5 per cent trichloroacetic acid per tube. 10  $\gamma$  of activated protein B were then added to the first tube (Fig. 6, A).

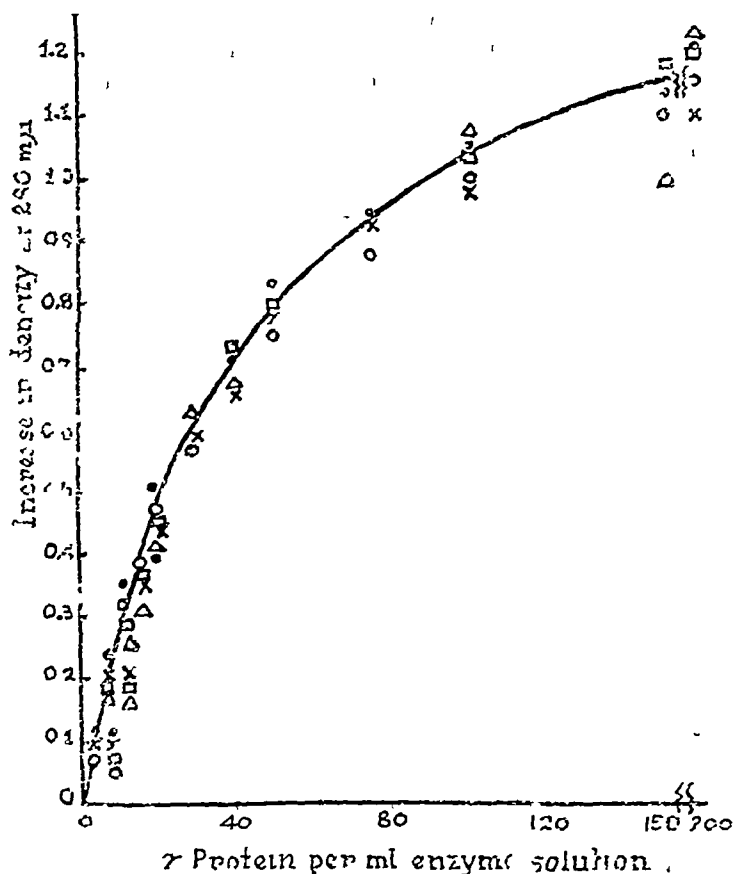


FIG. 1. Constant activity of crystalline activated protein B through several consecutive recrystallizations: ●, first recrystallization of Sample 1, △, second recrystallization of Sample 1, ○, third recrystallization of Sample 1, □, first recrystallization of Sample 2, ×, second recrystallization of Sample 2.

An analogous experiment with 100  $\gamma$  of activated protein B for the initial digestion of 40 minutes and 100  $\gamma$  of the three respective enzymes added for the additional digestion of 40 minutes are shown in Fig. 5, C. The experiment of this group is identical to the latter, with the exception that the initial digestion was carried out with 100  $\gamma$  of trypsin (Fig. 6, B).

Finally, an analogous experiment was performed in which the initial digestion of 40 minutes was carried out with 100  $\gamma$  of chymotrypsin. The three enzymes were then added and the digestion was continued for 40 minutes (Fig 6, D). Only the curve representing the addition of chymo-

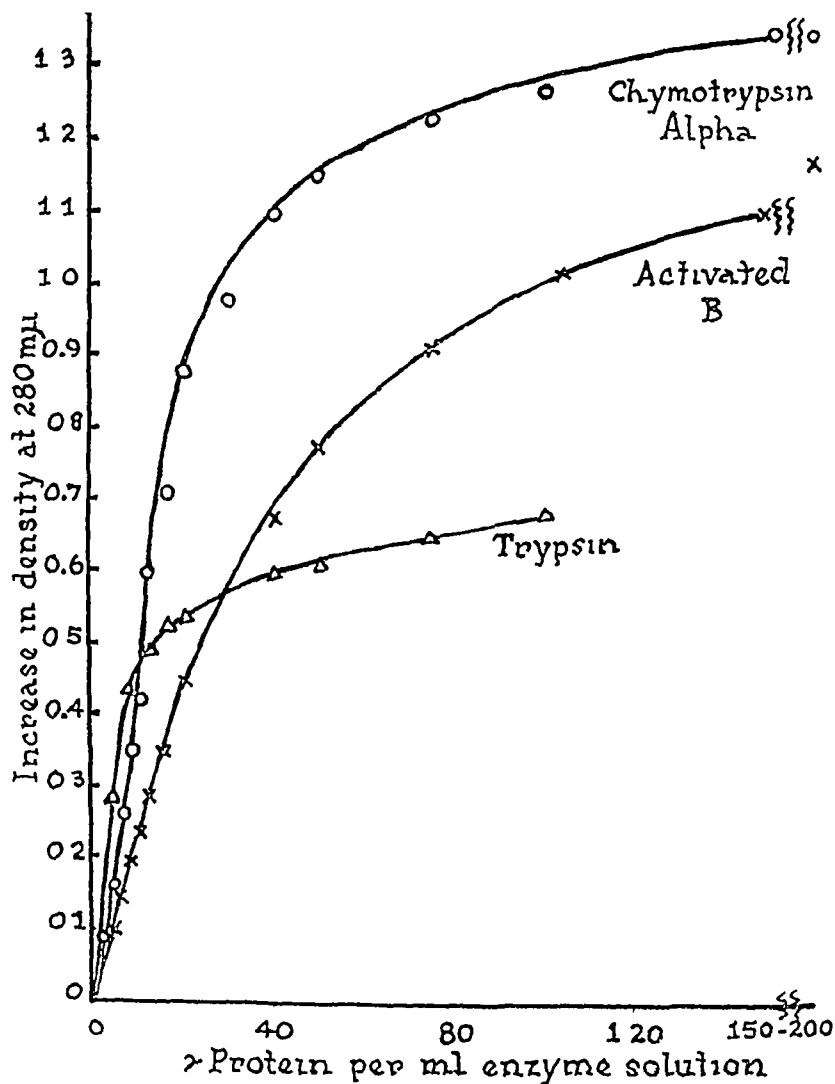


FIG 5 Comparison of activity of crystalline trypsin, crystalline chymotrypsin, and crystalline activated protein B

trypsin is shown, since the other values were too close to be clearly represented graphically. The results of these experiments showed that (1) the final state of digestion of casein by chymotrypsin, resulting in an optical density of approximately 1.400, could not be reached by the digestion

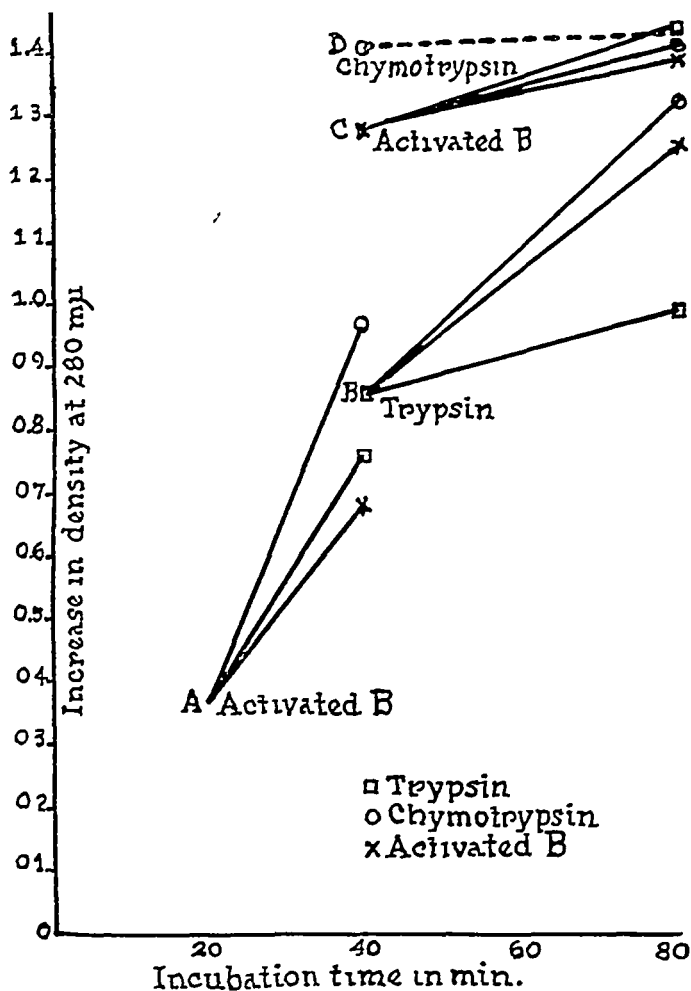


FIG 6 Effect of the addition of different enzymes to the partially digested casein. A, 20 minutes digestion with 10  $\gamma$  of activated protein B, followed by 20 minutes digestion with 10  $\gamma$  of trypsin, chymotrypsin, and activated protein B respectively B, 40 minutes digestion with 100  $\gamma$  of trypsin, followed by 40 minutes digestion with 100  $\gamma$  of trypsin, chymotrypsin, and activated protein B respectively C, 40 minutes digestion with 100  $\gamma$  of activated protein B, followed by 40 minutes digestion with 100  $\gamma$  of trypsin, chymotrypsin, and activated protein B respectively D, 40 minutes digestion with 100  $\gamma$  of chymotrypsin, followed by 40 minutes digestion with 100  $\gamma$  of chymotrypsin

with trypsin even when it was present in excess (Fig 6, B, trypsin), that (2) with sufficient excess of enzyme, both chymotrypsin and activated



protein B reached the same stage of digestion (Fig 6, *C, D*), and that (3) the rates of reaction of chymotrypsin and activated protein B were significantly different in all cases

According to Kunitz's method (4) for the determination of specific light absorption at  $280\text{ m}\mu$ , his factors for trypsin, 0.585, and chymotrypsin, 0.500, were confirmed. The factor for activated protein B was found to be 0.600.

Finally, attempts were made to crystallize chymotrypsin by the procedure described for activated protein B. To date all attempts have been unsuccessful. It was found, however, that chymotrypsinogen can be easily crystallized by the procedure described for unactivated protein B<sub>2</sub>. (2) Six recrystallizations of chymotrypsinogen by this method resulted in a formation of typical chymotrypsinogen needles, as shown by Kunitz and Northrop (5), with no tendency toward plate formation.

From the accumulated evidence it was concluded that the activated protein B belongs to the group of chymotrypsins. Direct comparison with chymotrypsin  $\alpha$  and comparison with the data available for chymotrypsins  $\beta$  and  $\gamma$  led to the conclusion that activated protein B was not identical with any of these enzymes. It was decided to name activated protein B chymotrypsin B, in preference to chymotrypsin  $\delta$ , in order to stress the different rates of reaction with casein. Similarly, since neither chymotrypsin  $\beta$  nor chymotrypsin  $\gamma$  has a known zymogen, it was felt advisable to make this choice rather than to extend the Greek series. Therefore, the unactivated protein B may be conveniently named chymotrypsinogen B.

#### SUMMARY

A method of crystallization of activated protein B has been described. The protein thus obtained maintained constant proteolytic activity through several successive recrystallizations. It was concluded that this crystalline substance represented a uniform proteolytic enzyme.

From the study of the proteolytic action of this enzyme by the digestion of casein, as well as from its ability to clot milk, and particularly on the evidence of Dr. Futon's work, it was concluded that this enzyme belongs to the group of chymotrypsins.

From the comparison of the rates of digestion of casein and the coefficients of light absorption at  $280\text{ m}\mu$  it was concluded that activated protein B is not identical with chymotrypsin  $\alpha$ .

The name chymotrypsin B was suggested for the activated form and chymotrypsinogen B for the unactivated form.

The authors wish to thank Mr L C Massopust for the photographs and drawings accompanying this paper

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## SPECIFICITY OF CHYMOTRYPSIN B

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(Received for publication, November 29, 1947)

Brown, Shupe, and Laskowski (1) have described the isolation, from beef pancreas, of a crystalline proteolytic enzyme which they have named "activated protein B" or "chymotrypsin B." Through the kindness of Dr. Laskowski who provided us with a 4 times recrystallized sample of this enzyme, it was possible to examine its action on a series of synthetic peptides and peptide derivatives. The data presented in Table I show that, of the compounds tested as possible substrates, only carbobenzoxy-L-tyrosylglycinamide, carbobenzoxyglycyl-L-tyrosylglycinamide, glycyl-L-tyrosinamide, glycyl-L-phenylalaninamide, L-tyrosylglycinamide, and L-phenylalanylglycinamide are hydrolyzed to an appreciable extent. It has been reported in previous communications (2, 3) that the substances named above are substrates for the crystalline chymotrypsin of Kunitz and Northrop (4), and that the site of hydrolysis is, in each case, at the peptide linkage involving the carbonyl group of the aromatic amino acid residue. In addition, earlier experiments (2) have shown no difference in specificity toward synthetic substrates between chymotrypsin and  $\gamma$ -chymotrypsin, the latter enzyme having been prepared by Kunitz (5) from chymotrypsin.

In contrast to the action of activated protein B on the substrates for chymotrypsin, no hydrolysis was found in the case of benzoyl-L-argininamide, which is a typical synthetic substrate for crystalline pancreatic trypsin. A similar negative result was noted for carbobenzoxyglycyl-L-phenylalanine, a substrate for crystalline pancreatic carboxypeptidase.

The above data on the specificity of activated protein B support the view, expressed by Brown, Shupe, and Laskowski, that this enzyme belongs to the group of the chymotrypsins.

### SUMMARY

The crystalline proteinase activated protein B of Brown, Shupe, and Laskowski catalyzes the hydrolysis of peptide derivatives previously found to be substrates for the crystalline chymotrypsin of Kunitz and Northrop. This result supports the conclusion that activated protein B is closely related to chymotrypsin.

TABLE I

*Action of Activated Protein B on Synthetic Substrates*

Substrate concentration, 0.05 M in the case of the soluble compounds. 0.125 mm of the sparingly soluble substrates was weighed into 2.5 cc volumetric flasks in which the enzymatic hydrolysis was performed. Enzyme concentration, 0.25 mg of protein nitrogen per cc of test solution, temperature, 38°, pH 7.3 to 7.6 (0.02 M veronal buffer).

Substrate	Time	Hydrolysis*
	hrs	per cent
Carbobenzoxy-L-tyrosylglycinamide†	4	28
	20	71
Carbobenzoxyglycyl-L-tyrosylglycinamide†	4	33
	20	74
Glycyl-L-tyrosinamide acetate	4	42
	20	77
Glycyl-L-phenylalaninamide acetate	4	14
	20	42
L-Tyrosylglycinamide acetate	4	12
	20	29
L-Phenylalanylglycinamide acetate	4	10
	20	30
Benzoyl-L-argininamide hydrochloride	20	2
Carbobenzoxyglycyl-L-phenylalanine	20	1
Carbobenzoxy-L-glutamyl-L-glutamic acid	20	2
Benzoylglycinamide†	20	0
Carbobenzoxy-L-isoglutamine	20	2
Carbobenzoxy-L-serinamide	20	0
Glycylglycylglycine	20	1

\* The data in this column are expressed as per cent of the hydrolysis expected on the complete hydrolysis of one peptide linkage. The procedure employed in the determination of the extent of hydrolysis was the same as that described earlier (2).

† This substance was not in solution at the start of the experiment.

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# THE EFFECT OF METHYLTESTOSTERONE ON THE RATE OF SYNTHESIS OF CREATINE\*

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After the daily administration of methyltestosterone to normal human subjects for several weeks, creatine usually appears in the urine in gradually increasing amounts (1, 2). Because the creatinuria so induced is associated with an increase in the concentration of serum creatine, and also because the amount of urinary creatinine increases, it has been generally assumed that the synthesis of creatine is accelerated by methyltestosterone.

The effect of methyltestosterone on the metabolism of creatine can be more directly observed by measuring the rate of turnover of creatine by isotope methods. It has previously been shown that the rate of dilution of labeled body creatine, as reflected in the concentration of isotope in urinary creatinine, is a measure of the rate of replacement of depot creatine by endogenous creatine (3-5). By so tagging the body creatine of a normal male subject subsisting on a diet as free of creatine and creatinine as possible, the rate of synthesis of creatine before and during the administration of methyltestosterone has been measured. Evidence will be presented that methyltestosterone does increase the rate of synthesis of creatine. That this is specifically brought about by its effect on the reaction or reactions involved in the formation of guanidoacetic acid is indicated by other data.

## EXPERIMENTAL

2 weeks before the administration of isotopic creatine the subject (H D H) was placed on a diet free of meat or meat products. Milk consumption was limited to 250 ml per day. The creatine of the body was then labeled with isotopic nitrogen by the intravenous administration of 1.67 gm of creatine (19.4 mg per kilo) containing 31 atom per cent excess  $N^{15}$  and 1.5 gm of guanidoacetic acid (17.5 mg per kilo) containing 30.5 atom per cent excess  $N^{15}$ , both in the  $\alpha$  position. The isotopic creatine was synthesized as described (5, 6), the  $N^{15}$  guanidoacetic acid was pre-

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pared by the method of Bland and Brand (7) Isolation of urinary creatinine for the analysis of  $N^{15}$  was begun on the 5th day after the isotopic creatine was given, and from that time every 7th day for 49 days During the 6 intervening days of each period, urine specimens were pooled and analyzed for creatine, creatinine, and guanidoacetic acid The method of Peters (8) was used for the measurement of creatine and creatinine, and that of Hoberman (9) for guanidoacetic acid The isolated creatinine was degraded to sarcosine, as described (5) The sarcosine, isolated as the toluene sulfonyl derivative, was analyzed for  $N^{15}$  in a mass spectrometer constructed according to the specifications of Nier (10)

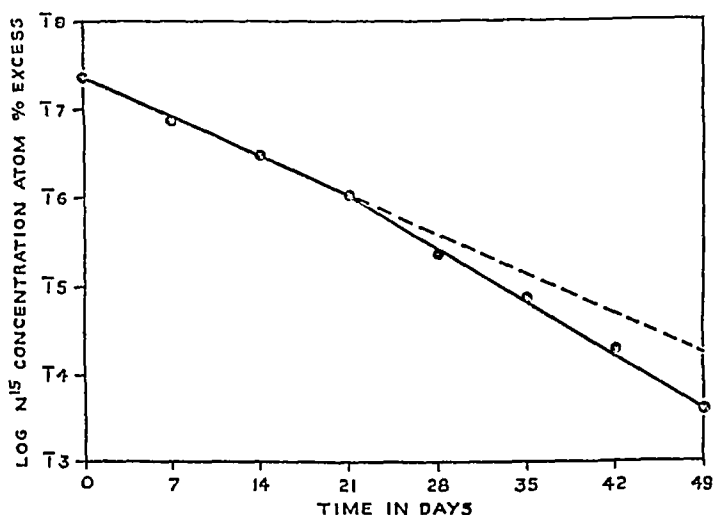


FIG 1 Changes in the isotope concentration of urinary creatinine observed before and during the administration of methyltestosterone The broken line is an extrapolation of the curve obtained during the control period

A control period of 20 days followed the day on which the first sample of creatinine was isolated Thereafter 50 mg of methyltestosterone were taken daily by mouth for 28 consecutive days

### Results

The changes in the isotope concentration of the urinary creatinine during the control period of 21 days and during the ensuing 28 days on methyltestosterone are shown in Fig 1 The slope of the straight line from  $t = 0$  to  $t = 21$ , obtained by plotting the data semilogarithmically, is 0.0143 This has been shown to be the fraction of the total body creatine which undergoes replacement each day (5) Since the average creatinine excretion during the control period was 1.76 gm per day, equivalent to 2.04 gm of creatine per day, the average total body creatine during this period is calculated to be  $2.04/0.0143 = 143$  gm

After methyltestosterone has been taken for 1 week, the slope of the curve of Fig 1 becomes steeper, denoting a more rapid rate of dilution of body creatine than during the control period. In a previous paper (5) it was shown that, for the condition of a changing total body creatine, labeled with isotope, the amount of creatine at any time  $t$  can be calculated by the substitution into the equation

$$G = \frac{C_0 G_0}{C} e^{-kt}$$

where  $C_0$  and  $C$  are the isotope concentrations of the urinary creatinine at time  $t = 0$  and  $t$ , respectively,  $G_0$  and  $G$  are the amounts of total body creatine at  $t = 0$  and  $t$ , respectively, and  $k$  is the turnover constant. For the application of this equation to the present study it has been assumed that methyltestosterone does not alter the fraction of the total body creatine which undergoes conversion to creatinine. With  $C_0 = 0.400$  atom per

TABLE I  
*Changes in Body Creatine Observed during Ingestion of Methyltestosterone*

Time	N <sup>15</sup> in creatinine	Total body creatine
days	atom per cent excess	gm
0	0.400	143
7	0.345	150
14	0.307	152
21	0.268	158
28	0.230	167

cent excess (the N<sup>15</sup> concentration of the urinary creatinine isolated on the last day of the control period),  $G_0 = 143$  gm and  $k = 0.0143$  per day,  $G$  has been calculated for the 28 day period, during which methyltestosterone was administered. The results of these calculations are shown in Table I.

It is apparent that the body depots gained 24 gm of creatine in the period during which methyltestosterone was taken. The average rate of synthesis of creatine therefore increased approximately 40 per cent.

Graphical integration of the data of the last column in Table I permits calculation of the average expected creatinine excretion according to

$$\bar{e} = \bar{I}\bar{G}$$

where  $\bar{e}$  is the average expected creatinine excretion (expressed as gm of creatine per day), and  $\bar{G}$  is the average number of gm of creatine in the body, the integration being performed over the 28 day period during which methyltestosterone was given. By this method  $\bar{e}$  was calculated to be



2.19 gm per day The average observed creatinine excretion during the same time interval was 1.85 gm per day, equivalent to 2.14 gm of creatine per day The agreement between the calculated and observed creatinine excretion supports the assumption that during the period under observation methyltestosterone was without effect on the process concerned with the transformation of creatine to creatinine

Although creatine could not be detected in the urine at any time during the experimental period, the urinary excretion of guanidoacetic acid increased approximately 70 per cent while methyltestosterone was administered This is shown in Fig 2 The rise in the excretion of urinary guanidoacetic acid which occurred during the control period is not significant,

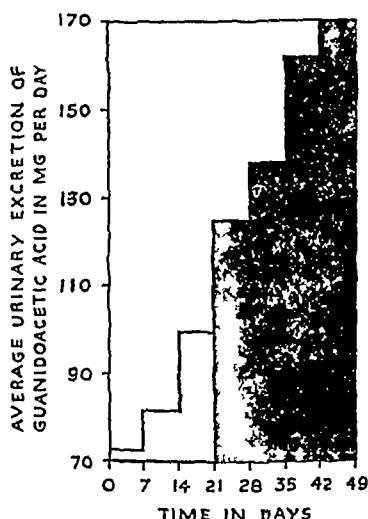


FIG 2 Changes in the urinary excretion of guanidoacetic acid before and during the administration of methyltestosterone The solid area represents the period during which methyltestosterone was taken

since variations of this order of magnitude have not been uncommon in our experience However, normal male urine has never been found to contain such large amounts of guanidoacetic acid as were excreted during the administration of methyltestosterone

#### DISCUSSION

It is significant that the rate of creatine synthesis increased during the 1st week of methyltestosterone administration The fact that several weeks usually elapse before methyltestosterone induces creatinuria is believed to be related to the amount of creatine already present in the body tissues Samuels *et al* (2) found it possible to shorten this latent period by incorporating extra creatine in the diet The absence of a detectable amount

of creatine in the urine after 4 weeks of ingestion of methyltestosterone can be attributed to the omission of creatine from the diet

A rise in the amount of guanidoacetic acid excreted in the urine of normal children has been reported by Hoagland *et al* (11) to follow the administration of methyltestosterone. The excretion of greater amounts of guanidoacetic acid during the period of administration of methyltestosterone than during the control period indicates that the step which determines the rate of synthesis of creatine, at least under the conditions brought about by the ingestion of methyltestosterone, is related to the methylation of guanidoacetic acid. Borsook *et al* (12) have presented evidence that guanidoacetic acid is formed in the kidneys in the human. Methyltestosterone administered to patients with severe nephritis induced neither creatinuria nor a significant rise in serum creatine (2). These observations are consistent with the hypothesis that methyltestosterone increases the rate of formation of creatine specifically by its effect on the synthesis of guanidoacetic acid. There is therefore no necessity of postulating that methyltestosterone acts in any way as a "methylating catalyst" (13).

That the rate of creatine turnover is independent of the methionine content of the diet has been observed by Cohn *et al* (4). It would appear that the rate of synthesis of creatine is determined under their conditions either by the rate of synthesis of guanidoacetic acid or by the concentration of the enzyme or enzymes available for the process of methylation. Further it has been observed in the human that oral tolerance to guanidoacetic acid is not increased by the administration of an equivalent amount of methionine<sup>1</sup>. It would therefore seem most probable that during the administration of methyltestosterone the rate of synthesis of creatine is limited by the concentration of catalysts required for the methylation of guanidoacetic acid.

The authors wish to acknowledge the capable technical assistance of Mrs. Marta Tobey and to thank Mr. Joseph Doolittle for performing the isotope analyses.

#### SUMMARY

The effect of methyltestosterone on the rate of synthesis of creatine has been studied in a normal human subject by means of isotope methods. Evidence is presented that methyltestosterone promptly brought about an increase in the rate of synthesis of creatine. That this is specifically due to the effect of methyltestosterone on the reaction or reactions involved in the synthesis of guanidoacetic acid is indicated by other data.

<sup>1</sup> Unpublished observations.

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# SOME PROPERTIES OF AN UNIDENTIFIED CHICK GROWTH FACTOR FOUND IN CONDENSED FISH SOLUBLES\*

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In previous papers (1, 2) evidence was presented to show that condensed fish solubles produced a marked increase in the growth rate of chicks on diets containing an adequate level of vitamins and essential amino acids. Berry, Carrick, Roberts, and Hauge (3) found that a water extract of fish solubles was also active in stimulating the growth of chicks. Very little other information on the properties of the unidentified growth factor in fish solubles is available. The present investigation was undertaken to study some of the properties of the factor.

## EXPERIMENTAL

The chicks used were day-old straight run (New Hampshire ♂♂ X single comb white Leghorn ♀♀) cross-bred chicks which were the progeny of hens on Diet B-1 described previously (2). The experimental groups were housed in electrically heated batteries with raised screen floors. The chicks were individually wing-banded and were weighed at the beginning of the experiment and at weekly intervals thereafter. Feed and water were supplied *ad libitum*. The experiments were terminated at the end of 4 weeks. The basal ration was the same as that reported in previous studies (1, 2) and consisted of ground yellow corn 35, wheat bran 10, wheat middlings 10, dehydrated alfalfa leaf meal 5, soy bean oil meal 28, vitamin test casein 7.5, limestone grit 2.0, steamed bone meal 1.5, iodized salt 0.5, fish oil (2000 vitamin A, 400 vitamin D units) 0.5, and manganese sulfate 0.025 gm, thiamine 0.3, riboflavin 0.6, niacin 5.0, calcium pantothenate 2.0, pyridoxane HCl 0.4, inositol 100.0, choline 150.0, *p*-aminobenzoic acid 10, biotin 0.02, folic acid 0.05, menadione 0.05, and  $\alpha$ -tocopherol 0.3 mg.

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Solubility of the factor was tested in water, 70 per cent MeOH, absolute MeOH, 70 per cent EtOH, 95 per cent EtOH, acetone, and ether. The water, alcohol, and acetone extracts were prepared in the following way. A weighed quantity of condensed fish solubles was mixed with sufficient solvent to result in the desired concentration allowing for the volume of water present in the fish solubles (50 per cent solids). The mixture was stirred mechanically for 1 hour at room temperature and filtered with suction through a Whatman No. 42 filter paper. The residue was reextracted twice with solvent of the desired concentration. The combined filtrates were concentrated and the organic solvents removed by distillation under reduced pressure. The residue remaining in the distillation flask was taken up in water.

The ether extraction was carried out for 24 hours with a liquid-liquid extractor which is a modification of the one described by Hossfeld (4).

Dialysis of fish solubles against distilled water was continued for 48 hours. Synthetic sausage casing membrane was used.

The extracts and the insoluble residues were mixed directly into the basal ration at a level equivalent to either 3 per cent or 6 per cent of the original condensed fish solubles.

The results are presented in Table I. In each trial the unsupplemented basal group and the group fed the basal ration supplemented with 3 per cent condensed fish solubles serve as negative and positive controls. The average weight of the chicks in each lot at 4 weeks of age is reported. A direct comparison of lots in different trials can be made by considering the per cent response which is calculated by considering the difference between the negative and positive controls as 100 per cent.

In confirmation of the observation of Berry *et al.* (3) the active principle was found in the water extract (Lots 3 and 12). Some growth-promoting activity remained in the residue (Lots 4 and 13). The growth factor was extracted by 70 per cent MeOH (Lots 18 and 32) and by 70 per cent EtOH (Lot 14). With these solvents the degree of extraction was comparable to that obtained with water. Filtration and distillation were facilitated by the alcohols. The absolute methanol extract (Lot 20) caused some promotion of growth but a greater part of the activity was present in the residue (Lot 21) in contrast to a lower concentration of this solvent.

The precipitate formed in 95 per cent EtOH at  $-4^{\circ}$  (Lot 28) contained full growth-promoting activity, and only very slight solubility of the factor was evident under these conditions (Lot 27). In acetone the factor was completely insoluble (Lot 25) and full activity was present in the residue (Lot 26).

Dialysates of condensed fish solubles (Lots 22, 35, and 39) gave a full response, indicating that the factor is readily dialyzable through synthetic sausage casing membrane. The growth stimulation obtained was of the

same order as that with water, 70 per cent MeOH, and 70 per cent EtOH extracts

TABLE I

*Average Weight and Comparative Response of Chicks to Various Fractions of Condensed Fish Solubles*

Trial No	Lot No	Supplement to basal ration		Average weight 4 wks	Response
			<i>per cent</i>		<i>per cent</i>
1	1	None		173	
	2	3% fish solubles		233	100
	3	H <sub>2</sub> O extract, fish solubles	≅6	247	123
	4	Residue, Lot 3	≅6	226	88
	5	Ether extract, fish solubles	≅6	163	-17
	6	Residue, Lot 5	≅6	246	+122
	7	As Lot 2, pH 1, autoclaved 20 mins, 15 lbs	≅3	171	-3
	8	As Lot 2, pH 7, autoclaved 20 mins, 15 lbs		198	42
	9	As Lot 2, pH 12, autoclaved 20 mins, 15 lbs		168	-8
2	10	None		232	
	11	3% fish solubles		307	100
	12	H <sub>2</sub> O extract	≅3	287	73
	13	Residue, Lot 12	≅3	250	24
	14	70% ethanol extract	≅3	285	71
	15	Residue, Lot 14		253	28
3	16	None		197	
	17	3% fish solubles		261	100
	18	70% methanol extract	≅6	278	126
	19	Residue, Lot 18	≅6	231	53
	20	Absolute methanol extract	≅6	266	108
	21	Residue, Lot 20	≅6	274	120
	22	Dialysate, fish solubles	≅3	264	105
4	23	None		209	
	24	3% fish solubles		298	100
	25	100% acetone extract	≅3	217	9
	26	Residue, Lot 25	≅3	306	108
	27	95% ethanol extract, -4°	≅3	241	35
	28	Residue, Lot 27, -4°	≅3	312	115
	29	Enzyme digest	≅3	306	108
5	30	None		192	
	31	3% fish solubles		300	100
	32	70% methanol extract	≅3	303	103
6	33	None		243	
	34	3% fish solubles		288	100
	35	Dialysate, fish solubles	≅3	284	91
	36	Residue, Lot 35		274	69

- 1 Soluble in water, 70 per cent methanol, 70 per cent ethanol
- 2 Somewhat soluble in absolute methanol and very slightly soluble in 95 per cent ethanol
- 3 Insoluble in ether and acetone
- 4 Dialyzable through a cellophane membrane
- 5 Heat-stable (100° for 2 hours) over a range of pH 3 to 9, no loss in activity was caused by enzymatic digestion

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# THE EFFECT OF SYNTHETIC PTERINS ON GROWTH AND HEMOGLOBIN FORMATION IN THE CHICK\*

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As early as 1936 Tschesche and Wolf (1) reported that certain pterins, especially xanthopterin, were effective in alleviating the anemia which develops in rats fed a goat's milk diet. Simmons and Norris (2) reported the hematopoietic effect of xanthopterin on an anemia in fingerling Chinook salmon which was produced by feeding a high protein diet. Totter and Day (3) found that xanthopterin had a partial effect in overcoming growth inhibition and leucopenia in the rat caused by the feeding of succinylsulfathiazole. Totter, Shukers, Kolson, Mims, and Day (4) reported that xanthopterin cured vitamin M deficiency in the monkey, and stated that their data suggested that xanthopterin is required by the monkey for normal hematopoiesis. On the other hand, O'Dell and Hogan (5) found that xanthopterin had no curative action on anemia in chicks fed a diet deficient in vitamin B<sub>6</sub>.

Thymine, when administered in large amounts, has been found by Spies, Vilter, Cline, and Frommeyer (6) to be effective in correcting certain human macrocytic anemias. Petering and Delor (7) have reported that thymine cannot substitute for folic acid in the nutrition of the rat.

The study reported in this paper was undertaken to determine the biological activity for the chick of a number of synthetic pterins and pyrimidines, several of which have been reported by Daniel and associates (8, 9) to have an inhibitory action on the growth of five species of bacteria. Approximately 60 compounds have been studied, but only those showing either vitamin or antivitamin activity will be discussed here.

## EXPERIMENTAL

All experiments were conducted with day-old white Leghorn cockerels which were housed in electrically heated batteries in a thermostatically controlled brooder house. All chicks were on wire mesh floors. Feed and

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D C, and was aided by grants to Cornell University by Cerophyl Laboratories, Inc., Kansas City, Missouri, the Nutrition Foundation, Inc., New York, and the Western Condensing Company, Appleton, Wisconsin. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry.

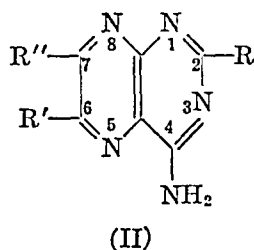
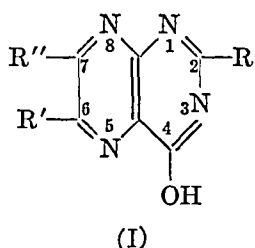


water were supplied *ad libitum*. Each treatment was started with fifteen chicks.

The chicks were fed purified Diet 653, as described by Hill, Norris, and Heuser (10). Microbiological assays, conducted upon the diet after it had been incubated with a folic acid conjugase preparation from fresh chick liver, showed that this basal diet contained approximately 2  $\gamma$  of folic acid per 100 gm.

The chicks were weighed individually at weekly intervals. Hemoglobin levels were determined at the end of the experiment (4 weeks) by the method of Robertson and Fiala<sup>1</sup> with 0.1 per cent  $\text{Na}_2\text{CO}_3$  and by measuring the oxyhemoglobin colorimetrically with a Coleman spectrophotometer.

*Compounds Studied*—The pterins and pyrimidines used in these experiments were synthesized by Cam and associates of the Chemistry Department, Cornell University. The syntheses of some of these compounds have already been reported (11, 12). The names and formulas of the compounds used are presented in Table I, for the simplification of which the two general structural formulas are presented.



*Experiment 1*—In this study, a factor S concentrate equivalent to 5 per cent dried brewers' yeast was added to the basal diet. This supplementation increased the folic acid content to 15  $\gamma$  per 100 gm of diet. *p*-Aminobenzoic acid (PABA) was omitted from the basal diet. Each compound was added to the diet at a level of 20 mg per 100 gm, with the exception of thymine, which was included at 500  $\gamma$  per 100 gm.

The results of Experiment 1 are presented in Table II. The data show that many of the pterins and pyrimidines caused a significant increase in weight, although not in hemoglobin level. One pterin, 2-amino-4-hydroxy-6,7-dimethylpteridine (Compound 14), had a marked inhibitory effect on growth and hemoglobin formation. Several compounds reduced the hemoglobin content, and in one case, 2-amino-4,6-dihydroxy-7-methylpteridine (Compound 18), increased the weight but decreased the hemoglobin level. Compounds 12, 13, 19, and 27 produced about a 50 gm increase in weight over that of the controls at 4 weeks of age. These differences proved to be

<sup>1</sup> Robertson, E. I., and Fiala, G. F., unpublished data.

highly significant when the data were analyzed statistically. Thymine caused a 30 gm increase in weight which was not statistically significant.

*Experiments 2 and 3*—For all subsequent studies it was decided to substitute crude for purified casein, to omit the factor S preparation, and to supply 15  $\gamma$  of synthetic folic acid per 100 gm of diet along with 0.01 per cent PABA.

TABLE II

*Growth and Hemoglobin Responses of Chicks to Synthetic Pterins and Pyrimidines*

Supplement Compound No	Average weight 4 wks	Average hemoglobin 4 wks	Statistical data	
			Weight	Hb
	gm	gm per 100 ml		
None*	175 (14)†	6.2		
50 $\gamma$ folic acid	305 (15)	8.4	Highly significant	Highly significant
3‡	185 (12)	5.6		
5	211 (15)	6.2	Significant	
6	215 (15)	5.7	"	
11	166 (11)	4.6		Significant§
12	220 (15)	5.4	Highly significant	
13	228 (15)	6.5	" "	
14	124 (12)	3.3	" " §	Highly significant§
18	216 (12)	4.3	Significant	Significant§
19	228 (13)	6.9	Highly significant	
22	176 (14)	5.5		
23	212 (15)	5.7	Significant	
24	217 (15)	6.0	"	
25	165 (14)	4.7		Significant§
26 (Thymine)	207 (14)	6.3		
27	232 (15)	7.0	Highly significant	
28 (Alloxan)	211 (14)	6.2	Significant	

\* The basal diet contained a crude preparation of factor S which brought the total amount of folic acid in the diet to 15  $\gamma$  per 100 gm.

† The numbers in parentheses represent the number of surviving chicks of the fifteen started.

‡ The compounds were added at a level of 20 mg per 100 gm of diet, with the exception of thymine, which was at 500  $\gamma$  per 100 gm.

§ Less than the controls.

The results of Experiments 2 and 3 are presented in Table III. The most striking effect produced by the compounds used in Experiment 2 was upon hemoglobin formation. The acid derivatives, especially the 7-carboxypteridines (Compounds 7 to 10), were the most effective. As found in Experiment 1, Compound 14, 2-amino-4-hydroxy-6,7-dimethylpteridine, caused a decrease in weight that was significantly different from that of the

controls. However, in this experiment the hemoglobin was unaffected by this compound.

The only pterin in Experiment 3 which had any effect was 2-amino-4-hydroxy-6,7-diphenylpteridine (Compound 20). It markedly decreased

TABLE III

*Growth and Hemoglobin Responses of Chicks to Several Series of Related Pterins*

Experiment No	Supplement Compound No	Average weight, 4 wks	Average hemo-globin, 4 wks	Statistical data	
				Weight	Hb
		gm	gm per 100 ml		
2	None*	196 (15)†	6.1		
	50 $\gamma$ folic acid	280 (15)	10.4	Highly significant	Highly significant
	3‡	204 (12)	6.4		
	4	188 (14)	7.1		Significant
	5	197 (14)	6.6		
	6	201 (13)	7.7		Highly significant
	7	238 (15)	8.3	Significant	" "
	8	217 (14)	9.8		" "
	9	203 (15)	7.7		" "
	10	225 (14)	7.6		" "
	14	152 (12)	5.5	Significant§	
	15	188 (14)	5.6		
	16	185 (13)	6.7		
	17	185 (14)	6.6		
3	None*	191 (11)	6.1		
	50 $\gamma$ folic acid	262 (15)	8.7	Highly significant	Highly significant
	1	168 (13)	5.2		
	2	176 (15)	5.5		
	20	107 (7)	3.8	Highly significant§	Significant§
	21	162 (9)	5.4		
	26 (Thymine)	155 (13)	5.6	Significant§	

\* The basal diet of both experiments contained 15  $\gamma$  per 100 gm of added synthetic folic acid.

† The numbers in parentheses represent the number of surviving chicks of the fifteen started.

‡ The compounds were included at the level of 20 mg per 100 gm of diet, including thymine.

§ Less than the controls.

growth and produced a significant lowering of the hemoglobin level. In this experiment thymine, at 20 mg per 100 gm of diet, decreased growth somewhat, but had no effect upon the hemoglobin level.

*Experiment 4*—Experiment 4 was designed to study the oral *versus* the

injected administration of 2,4-diamino-6,7-diphenylpteridine (Compound 21) and to repeat the feeding of 2-amino-4-hydroxy-6,7-diphenylpteridine (Compound 20). Crude casein, PABA, and 15  $\gamma$  of folic acid per 100 gm were included in the basal diet. All compounds administered orally were included at the level of 20 mg per 100 gm of diet. One lot received 50  $\gamma$  of Compound 21 dissolved in 0.5 ml of physiological saline by injection into the breast muscle of each chick twice a day for the 1st week. Thereafter, 1 ml of this solution was injected daily. At the end of the 4th week, approximately 1 ml of blood was removed from the wing vein of each chick in all lots. The blood from five chicks was pooled, and folic acid was

TABLE IV

*Effect of 2,4-Diaminopteridine (Compound 21) and Its 2-Amino-4-hydroxy Analogue (Compound 20) upon Growth, Hemoglobin Formation, and Folic Acid Content of Liver and Blood in Chicks*

Supplement Compound No	Average weight 4 wks	Average hemo- globin 4 wks	Folic acid in	
			Liver*	Blood†
	gm	gm per 100 ml	$\gamma$	mg per ml
None‡	238 (15)§	8.1	5.4	1.11
21 (injected)	244 (15)	7.4	3.0	0.68
21 (in feed)	242 (15)	7.3	2.9	0.62
20 " "	222 (15)	6.9	1.3	0.25
50 $\gamma$ folic acid	241 (14)	8.1	7.8	0.91

\* Average of ten livers per lot

† Every value is the average of three samples, each containing the pooled blood from five chicks

‡ The basal diet contained 15  $\gamma$  of added synthetic folic acid per 100 gm

§ The numbers in parentheses represent the number of surviving chicks of the fifteen started

|| This value is significantly lower than that of the control

determined on each pooled sample. The livers were removed from ten chicks in each lot and the amount of free folic acid was determined on each liver. Folic acid was determined by a modification (8) of the *Streptococcus faecalis* method of Luckey, Briggs, and Elvehjem (13). The samples of blood were diluted 1:2 with H<sub>2</sub>O and steamed for 30 minutes, after which they were filtered through Whatman No. 44 filter paper with Celite filter aid and suction. The livers were ground individually in a Waring blender in phosphate buffer at pH 7.0, centrifuged, made to the desired concentration, autoclaved for 15 minutes, and filtered.

The results of Experiment 4 are presented in Table IV. It is apparent from the data that the chicks grew better on the folic acid-deficient diet

in Experiment 4 than in Experiments 2 and 3. Despite the fact that the growth response in the lot receiving 50  $\gamma$  of folic acid per 100 gm of diet was not quite up to that obtained in the previous experiments, the microbiological data for this lot appear to be in accord with the rest of the data.

There were no differences in the results obtained when Compound 21 was injected as compared to its oral administration. Compound 20, 2-amino-4-hydroxy-6,7-diphenylpteridine, caused a significant decrease in hemoglobin level, but did not affect weight. The folic acid values for the liver and blood were decidedly less when either of the pterins was supplied. Compound 20, however, produced the greatest decrease in the liver and blood folic acid, these values being reduced in this case to approximately 25 per cent of those of the control lot.

*Experiment 5*—This study was conducted to determine whether or not the addition of a sulfonamide together with pterin, Compound 14, would increase or decrease the inhibitory effect of this pterin upon chick growth and hemoglobin formation. Information was also desired as to whether the inhibitory effect of the pterin alone could be neutralized by added folic acid. PABA was omitted from the diet of the chicks receiving the sulfonamide. A complete blood study was made on five chicks from the lot receiving 15  $\gamma$  of folic acid and on five chicks from the lot receiving 15  $\gamma$  of folic acid plus pterin No. 14. The results of the experiment are presented in Table V. As in Experiment 4, the growth and hemoglobin levels of the chicks receiving 15  $\gamma$  of folic acid per 100 gm of diet were unusually high. A possible explanation of this apparent reduction in folic acid requirement is that Experiments 4 and 5 were conducted with chicks from hens having access to the green range of early summer. Eggs from such hens may contain a larger storage of folic acid, which would allow chicks from these eggs to maintain a higher growth rate for the first few weeks on a folic acid-deficient diet.

Compound 14, 2-amino-4-hydroxy-6,7-dimethylpteridine, again significantly depressed growth and hemoglobin formation. The addition of 60  $\gamma$  of folic acid to the 20 mg of Compound 14 caused a 74 gm increase in weight and an increase in hemoglobin level of 1.4 gm per 100 ml of blood. However, these values, especially the hemoglobin level, are not quite normal. Evidently enough folic acid had not been added to counteract completely the inhibitory effects of the compound.

In Experiment 5, 2 per cent succinylsulfathiazole did not depress growth, but did decrease the hemoglobin level significantly. This is contrary to the earlier findings of Robertson, Daniel, Farmer, Norris, and Heuser (14). However, it may be explained by the fact that in the present work crude casein was used instead of the purified casein used earlier. Nevertheless, the addition of Compound 14 to 15  $\gamma$  of folic acid and 2 per cent sulfona-

mide did not alter the usual inhibitory effect obtained with this pterin. From these data it may be stated that the sulfonamide-sensitive bacteria in the intestinal tract of the chick were not responsible for the antagonistic action of this pterin.

The results of the blood study indicate that the addition of the compound caused a decrease in red blood cells, a decided lowering in hematocrit, and a resultant macrocytic anemia. The unusual responses to 15  $\gamma$  of folic acid per 100 gm of diet by these chicks were substantiated by the blood picture. The number of red blood cells was almost normal, as was

TABLE V

*Effect of Folic Acid and Sulfonamide on Action of 2-Amino-4-hydroxy-6,7-dimethylpteridine (Compound 14) in Chicks*

Supplement	Average weight 4 wks	Average hemoglobin, 4 wks	Average red blood cells	Average hematocrit	Average mean corpuscular volume
	gm	gm per 100 ml	millions per c mm	ml per 100 ml	cu microns
15 $\gamma$ folic acid	270 (14)*	9.5	2.26†	29	127
60 " " "	307 (15)	9.0			
15 " " " + pterin‡	208 (14)	5.6	0.97	14	148
60 " " " + "	282 (13)	7.0			
15 " " " + 2% sulfonamide§	255 (15)	7.0			
15 $\gamma$ folic acid + 2% sulfonamide + pterin	219 (13)	5.0			

\* The numbers in parentheses represent the number of surviving chicks of the fifteen started.

† The values in the last three columns are the averages of determinations on five chicks.

‡ Pterin added at 20 mg per 100 gm of diet.

§ Succinylsulfathiazole.

the hematocrit. The cells were slightly macrocytic when compared with those of the chicks receiving a higher level of folic acid reported in the Experiment 6.

*Experiment 6*—The purpose of Experiment 6 was to determine whether a high level of folic acid would overcome the antagonistic action of an inhibitory pterin, and to ascertain any differences resulting from injecting the compound, as compared to its oral administration. At the end of this experiment a complete blood study was performed on eight chicks from each lot. The results of Experiment 6 are presented in Table VI.

The addition of a relatively large amount of folic acid completely corrected the low hemoglobin level induced by the pterin, and restored prac-

tically normal growth. The slight difference in weight between the lot receiving the pterin plus 200  $\gamma$  of folic acid and the lot receiving 200  $\gamma$  of folic acid alone did not prove to be significant. The data on the blood picture verify the evidence obtained in Experiment 5 that the inhibitory effect of the pterin can be overcome by adding sufficient folic acid.

Oral administration of the pterin caused a somewhat more severe anemia than was obtained on the basal level of folic acid alone. The hemoglobin was decreased, as were the hematocrit and the number of red blood cells. The mean corpuscular volume, however, was unchanged. The folic acid

TABLE VI

*Effect of Injecting and Feeding 2-Amino-4-hydroxy-6,7-dimethylpteridine (Compound 14), Counteracting Effect of Folic Acid*

Supplement	Average weight, 4 wks	Average hemoglobin, 4 wks	Average red blood cells	Average hematocrit	Average mean corpuscular volume	Folic acid in blood*
	gm	gm per 100 ml	millions per c mm	ml per 100 ml	cu microns	mg per ml
15 $\gamma$ folic acid	151 (14)†	5.9	0.96‡	13‡	130‡	1.08
15 " " " + pterin (oral)§	99 (10)	4.2	0.88	11	131	0.63
15 " " " + pterin (injected)	127 (15)	5.2	1.26	18	142	1.07
200 $\gamma$ folic acid + pterin (oral)	241 (11)	9.9	2.60	29	112	4.67
200 " folic acid	272 (12)	10.3	2.42	28	114	3.75

\* Each value is the average of three samples containing pooled blood from four to five chicks.

† The numbers in parentheses represent the number of surviving chicks of the fifteen started.

‡ The values in these three columns are the averages of determinations on eight chicks.

§ Pterin added at 20 mg per 100 gm of diet.

|| Pterin injected intramuscularly at the level of 100  $\gamma$  per day.

content of the blood was decreased. The injection of the pterin at a level of 100  $\gamma$  per chick per day was much less effective in producing inhibition than when it was added to the feed. However, the amount of pterin consumed per chick per day in the feed was from 1400  $\gamma$  the 1st week to 2000  $\gamma$  the 4th week. The addition of 200  $\gamma$  of folic acid to the feed containing the pterin corrected all phases of the anemia.

#### DISCUSSION

The data presented in this report indicate that several pterins and pyrimidines were utilized by the chick for growth and hemoglobin formation on a folic acid-deficient diet. However, the responses were not maximum, even when the amount fed was approximately 400 times the folic acid

requirements for growth and hemoglobin formation in white Leghorn chicks. In the course of these studies, two pterins were found to inhibit growth and hemoglobin formation markedly when added to a diet containing a suboptimum level of folic acid.

The presence of folic acid in a suboptimum amount was found to be necessary to effect any response from the compounds. In preliminary experiments not reported here, several of the same pterins that showed a growth response in Experiments 1 and 2 were ineffective when supplementing the basal diet containing no added folic acid. The pterins that inhibited chick growth could not be expected to have an effect on this diet which is severely deficient in folic acid, except to increase mortality, since the weights of the surviving chicks were very low (100 gm. at 4 weeks). A possible explanation for the growth-promoting effect of several of the pterins on the diet containing a low level of folic acid only is that the intestinal bacteria, in the presence of folic acid, are able to grow and thereby convert these compounds into substances having folic acid activity for the chick. Without folic acid, however, this synthesis does not occur. However, it is doubtful whether much synthesis of folic acid takes place on this diet in the presence of 20 per cent succinylsulfathiazole. It is possible that other necessary precursors of folic acid are not present in the diet.

Greater increases in growth occurred when the crude factor S fraction was present in the diet than when synthetic folic acid was added at the same level as that supplied by the factor S preparation. It is doubtful whether this response can be attributed to factor S (strepogenin) itself, since in Experiments 2 and 3 crude casein was used, and this product contains factor S far in excess of the factor S requirement of the chick, as reported by Scott, Norris, and Heuser (15). It is possible that some other substance present in the crude preparation may have enabled the intestinal bacteria to synthesize more folic acid from these compounds.

It is of interest to compare the microbiological results obtained with some of these compounds with the results obtained with the chicks. The 2,4-diaminopterins markedly inhibited bacterial growth. On the other hand, these pterins had no effect on growth or hemoglobin formation in the chick. Two compounds, however, which consistently inhibited growth and hemoglobin formation, were 2-amino-4-hydroxy-6,7-dimethylpteridine (Compound 14) and 2-amino-4-hydroxy-6,7-diphenylpteridine (Compound 20). These pterins resemble the pteridine nucleus of folic acid in the 2 and 4 positions, and are therefore more nearly similar to the pterin of folic acid than are the 2,4-diaminopterins. In Experiment 4, the blood and liver folic acid values indicated that even the diaminopterin affected folic acid metabolism, although the 2-amino-4-hydroxy analogue was more effective. However, the fact must not be overlooked that the folic acid values were measured by *Streptococcus faecalis* assay, and this organism showed an antibacterial



index of 8 for this compound (8) All of the decrease in folic acid content of the blood and liver of chicks receiving this pterin may have been due to the presence of unchanged 2,4-diamino-6,7-diphenylpteridine, which inhibits the response of *Streptococcus faecalis* The 2-amino-4-hydroxy-6,7-diphenylpteridine does not cause inhibition of *Streptococcus faecalis* Thus the decrease in folic acid content of the blood and liver in this case was a direct effect It is very probable that this compound (Compound 20) and 2-amino-4-hydroxy-6,7-dimethylpteridine (Compound 14) are antifolic acid compounds in the metabolic processes of animals, since folic acid was capable of overcoming the inhibition The presence of either one in the blood may prevent the storage of folic acid in the liver as it is absorbed, or may cause a depletion of folic acid in the liver by demanding a greater release of the vitamin from the liver to compete with the foreign pterin

The diaminopterin were no more effective when injected than when administered orally In neither case did these pterins interfere with growth and hemoglobin formation The folic acid levels of the liver and blood of both lots were the same Therefore, the absorption of the compound must have occurred without alteration in the intestinal tract On the other hand, there was a difference in response between the injected and orally administered 2-amino-4-hydroxy compound This compound, when fed, was more effective in inhibiting growth and lowering hemoglobin level There may be two explanations for this Either the chicks were not getting enough of the compound by injection, or bacteria in the tract, not affected by sulfonamide, were needed for the action of the compound The former seems to be more plausible, since the chicks getting the compound in the feed consumed about 14 times as much of this pterin per day the 1st week and increasing amounts each succeeding week It is logical to suppose that the bacteria that are subject to sulfonamide action (folic acid-synthesizing organisms) would be the ones to synthesize an antifolic acid from the compound fed Yet, the addition of succinylsulfathiazole had no effect on the action of the pterin

It is evident from these experiments that the pterins which inhibited chick growth and lowered hemoglobin formation acted by producing a folic acid deficiency in the chick This deficiency was completely prevented by the addition of sufficiently large amounts of folic acid Although clear cut evidence was not obtained on the ratio of the inhibitor to vitamin (amount of inhibitor to amount of vitamin), it is possible to state that the range of this index lies between 800 and 1500

In general, the carboxyl derivatives had the greatest beneficial effect upon growth and hemoglobin formation These compounds also were active in promoting some growth of *Streptococcus faecalis* (8)

Thymine has been shown not to substitute for folic acid in the nutrition

of the chick From the results presented here and those of Petering and Delor (7) on the rat, thymine does not appear to function in these animals in the same manner as it does in man (6)

#### SUMMARY

Several synthetic pterins and pyrimidines have been shown to bring about increased growth and hemoglobin formation in the chick Two pterins, 2-amino-4-hydroxy-6,7-dimethylpteridine and 2-amino-4-hydroxy-6,7-diphenylpteridine, have demonstrated an inhibitory action which was counteracted by additional folic acid That the inhibition was due to interference with folic acid metabolism was further indicated by the fact that greatly reduced levels of this vitamin were found in the liver and blood after the administration of the pterin Sulfonamides had no effect on this inhibitory action

Thymine possessed no folic acid activity for the chick, even when fed at high levels

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# A COMPOSITE BASAL MEDIUM FOR THE MICROBIOLOGICAL ASSAY OF LEUCINE\*

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The convenience and utility of microbiological procedures for the determination of amino acids would be increased if a single mixture containing all of the components of each basal medium could be compounded. It might be anticipated that such a mixture would be unstable, owing to interactions between glucose, amino acids, and possibly other of the nutrient substances, but there appears to be no record of any experimental work on this problem. Rockland and Dunn (1) have described the preparation in a ball mill of mixtures containing as many as eighteen amino acids which were not noticeably hygroscopic, were stable for at least 6 months, and were employed successfully in the determination of seven amino acids. Such mixtures are being used routinely in the authors' laboratory for the determination of nearly all of the common amino acids.

A composite basal medium for the assay of leucine has been prepared and stored for 8 months (a) at room temperature in a paraffin-sealed container, (b) at room temperature in a screw-capped jar, and (c) at about 5° over anhydrous calcium chloride. The powders were colored slightly yellow and the solutions deeper yellow, due primarily to riboflavin. The solutions yielded by the freshly prepared amino acids and the composite basal medium stored in the refrigerator had identical appearance, while that of the medium stored in a sealed container was slightly, but perceptibly, darker colored. The solution of the medium stored in a screw-capped jar was somewhat darker than the others. The mixtures darkened rapidly (in 2 days at 40° and in 2 hours at 70°) in accelerated storage tests. The composite medium is readily preserved under anhydrous conditions but, in the presence of moisture, it changes rapidly to a dark sticky mass.

It was found, on tests of these preparations described in the "Experimental" part, that assays of casein, silk fibroin, and two amino acid tests mixtures gave results for leucine which were identical within the stated experimental error to those obtained with a freshly prepared control basal

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medium It seems probable that similar basal media could be prepared which would be equally satisfactory for the determination of other amino acids

TABLE I  
*Composition of Basal Medium\**

Constituent	Weight	Constituent	Weight
	<i>gm</i>		<i>gm</i>
Glucose	200	DL-Norleucine†	0 92
Sodium acetate	120	DL-Norvaline†	0 92
Ammonium chloride	40	DL-Phenylalanine	2 29
KH <sub>2</sub> PO <sub>4</sub>	5 5	L-Proline†	0 46
K <sub>2</sub> HPO <sub>4</sub>	5 5	DL-Serine†	0 92
MgSO <sub>4</sub> 7H <sub>2</sub> O	2 2	DL-Threonine	0 92
FeSO <sub>4</sub> 7H <sub>2</sub> O	0 11	DL-Tryptophan	0 46
MnSO <sub>4</sub> 4H <sub>2</sub> O	0 11	L-Tyrosine	0 92
NaCl	1 0	DL-Valine	3 43
DL-Alanine†	4 58		<i>mg</i>
L-Asparagine†	0 46	Adenine sulfate 2H <sub>2</sub> O	145
L-Arginine HCl	4 58	Guanine HCl 2H <sub>2</sub> O	137
L-Cysteine HCl	0 915	Uracil	132
L-Glutamic acid	9 15	Thiamine HCl	11
Glycine	0 46	Pyridoxine	18
L-Histidine HCl H <sub>2</sub> O†	0 46	Calcium-dl-pantothenate	22
DL-Isoleucine	4 58	Riboflavin	22
DL-Lysine HCl†	0 92	Nicotinic acid	22
DL-Methionine	0 46	p-Aminobenzoic acid	1 1
			<i>γ</i>
		Biotin	55
Total			415 gm

\* The proportions of the amino acids were established (unpublished experiments) on the basis of the nutritional requirements of *Lactobacillus arabinosus* 17-5 determined by Dunn *et al* (2)

† Although this amino acid has been shown to be non-essential for *Lactobacillus arabinosus* 17-5 (2), it has been included in the basal medium because of the possibility that it might be stimulatory at low levels of leucine

#### EXPERIMENTAL

The composite basal medium was prepared by placing the quantities of nutrients designated in Table I in a dry ball mill jar, adding dry stones to the jar, and drying the mixture overnight in a vacuum desiccator over anhydrous calcium chloride. The lid of the jar was sealed with paraffin and the mixture was milled for 20 hours. Although the resulting fine powder was quite hygroscopic, it was readily maintained free flowing when

preserved in a tightly stoppered bottle. The composite medium was readily and completely soluble in cold water, except for a trace of insoluble material presumably derived from the ball mill stones.

The assays were performed as previously described (3). The tubes were incubated for 3 days at 34° and the resulting acid was titrated with standard base with bromothymol blue as indicator. Each 4 inch test-tube contained 3 ml final volume of solution equivalent to 124.5 mg of the composite basal

TABLE II  
*Summary of Leucine Assay Data\**

Medium	Experiment No	Casein hydrolysate No		Silk fibroin hydrolysate No		Gelatin hydrolysate No		Amino acid Test Mixture	
		1	2	1	2	1	2	A	B
a	1	9.6	9.6	0.83	0.87	3.3	3.3	104	103
	2	9.4	9.3	0.83	0.83	3.2	3.3	100	
	3	9.9	9.7	0.86	0.89	3.4	3.4	104	112
Average		9.6 ± 0.2		0.85 ± 0.02		3.3 ± 0.06		102	107
b	1	9.6	9.5	0.79	0.83	3.3	3.3	103	104
	2	9.5	9.5	0.82	0.84	3.3	3.3	103	
	3	9.9	9.7	0.84	0.84	3.4	3.4	105	106
Average		9.6 ± 0.1		0.83 ± 0.02		3.3 ± 0.06		104	105
c	1	9.5	9.3	0.76	0.79	3.2	3.2	98	100
	2†			0.85	0.77				

\* Values given as per cent of the moisture- and ash-free proteins or as per cent recovery from amino acid test mixtures. The average values are given as per cent plus or minus the mean deviation from the mean. Medium a = the freshly prepared mixture of amino acids, Medium b = the composite basal medium stored for 8 months at room temperature in a paraffin sealed jar, Medium c = the composite basal medium stored for 8 months at room temperature in a screw-capped jar which was opened at intervals during the storage period. Test Mixture A simulates casein, Test Mixture B, gelatin.

† The available material was insufficient for additional determinations.

medium. The samples were run at five levels, each in triplicate. The results are given in Table II.

#### SUMMARY

A dry, composite basal medium has been prepared which, after storage for 8 months at 5° and room temperature, was shown to be satisfactory for the determination of leucine with *Lactobacillus arabinosus* 17-5. It has

been suggested that similar basal media could be employed for the determination of other amino acids

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# GLUCOSE CONTENT OF THE BODY FLUID IN MARINE ANNELIDS

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Bahl (1) maintains that there are 100 mg of glucose in 100 cc of earthworm blood but that there is no sugar in the coelomic fluid. On the other hand, Damboviceanu (2) found no reducing sugar in the plasma from *Sipunculus nudus*. There is no information in the literature concerning the sugar content of the body fluid in marine annelids from the American coast. A series of analyses was, consequently, made to ascertain the amount of glucose in two common North American marine worms.

## Material and Methods

The worms, *Phascolosoma gouldii* and *Amphitrite ornata*, were obtained in the living condition from the supply department of the Marine Biological Laboratory during the summer of 1947. Body fluid was carefully removed with a hypodermic needle and syringe. Only fresh samples were studied. The amount of glucose in each sample was estimated according to the method of Folin (3). A second series of analyses was made by the method of Somogyi (7). Only the latter method was used for the body fluid from *Amphitrite*. Another series of analyses was made of fluid from *Phascolosoma* which had been heated to 30° for 30 minutes.

## Results

The results are summarized in Table I which shows that in *Phascolosoma* there is an average of 17.3 mg of glucose per 100 cc of fluid with the Folin filtrate, but only an average of 4.6 mg per 100 cc with the Somogyi filtrate. In *Amphitrite* the Somogyi filtrate yields an average of 8.4 mg of glucose per 100 cc of body fluid. After heating *Phascolosoma* to 30° for 30 minutes, there is a marked increase in the glucose values with either filtrate. Folin, 30.2 mg per 100 cc, Somogyi, 26.1 mg per 100 cc.

## DISCUSSION

The results show that in these two marine worms there is much less glucose than in human blood (70 to 90 mg per 100 cc) or in earthworm blood (100 mg per 100 cc). The values are, however, similar to those



obtained for *Sipunculus*, which contains 10 to 25 mg of glucose per 100 cc of body fluid (4)

A comparison of the present results with those of Morgulis (5), who studied the blood in *Homarus*, *Libinia*, *Callinectes*, and *Limulus*, and with those of Myers (6), who analyzed blood from *Strongylocentrotus*, *Cancer*, and *Haliotis*, indicates that the body fluid in the annelids contains appreciably less glucose than the fluid from other invertebrates

*Arenicola marina* is another marine annelid which is reported to have a relatively low content of sugar in the coelomic fluid (8)

TABLE I

*Amount of Glucose in Phascolosoma gouldii and in Amphitrite ornata*

Glucose values (in mg per 100 cc of body fluid) are given for the *Phascolosoma* at 20° and after heating to 30° for 30 minutes

Sample No	<i>Phascolosoma gouldii</i>				<i>Amphitrite ornata</i>
	Folin filtrate		Somogyi filtrate		Somogyi filtrate
	20°	30°	20°	30°	
1	15	30	5	27	10
2	15	32	7	27	10
3	22	27	5	25	5
4	15	32	3	27	10
5	15	30	3	25	7
6	22		5	27	
7	20		4	25	
8	15		4		
9	15		6		
10	19		4		
Mean	17.3	30.2	4.6	26.1	8.4

The increase in sugar content of the body fluid in *Phascolosoma* after heating at 30° for 30 minutes is interesting. Apparently, the increased temperature stimulated the breakdown of glycogen stored in the muscles or elsewhere. Since nothing is known about the mobilization and storage of carbohydrates in *Phascolosoma*, the place of origin of the increased amount of sugar in the body fluid after heating is not at all certain. A change in the relation of sugar values obtained with the Folin filtrate to those obtained with the Somogyi filtrate occurred after heating. At 20° the relation of Somogyi filtrate sugar to Folin filtrate sugar is 0.28, after heating to 30° for 30 minutes the relation of these quantities is 0.86. These two ratios indicate that there is a real increase in glucose values in *Phascolosoma* after heating and not chiefly of non-glucose reducing substances.

## SUMMARY

1 The amounts of glucose in the body fluids of *Phascolosoma gouldi* and *Amphitrite ornata* were estimated

2 It was found that there is an average of 4.6 mg of true glucose per 100 cc in the fluid from *Phascolosoma* and 8.4 mg per 100 cc in *Amphitrite*

3 After heating *Phascolosoma* to 30° for 30 minutes the amount of true glucose in the body fluid increases to 26.1 mg per 100 cc

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# THE PREPARATION OF A SOLUBLE CYTOCHROME OXIDASE\*

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Numerous attempts have been made to prepare a soluble cytochrome oxidase. Most recently, Haas (1) has reported a soluble oxidase prepared from heart muscle by first grinding and autolyzing and by then exposing the preparation to ultrasonic radiation. If true solution was not achieved, at least the particles were reduced in size. Later (2) he indicated that the complex could be separated into two components by centrifugation at 10,000 R P M for 2 hours.

The use of bile salts to extract or peptize insoluble heart muscle preparations (3, 4) suggested to us that the oxidase complex might be dissolved in this way. Our experiments indicate that Keilin and Hartree's insoluble oxidase complex can be dissolved with the aid of sodium desoxycholate (5). The resulting preparation is much more active than the original suspension and retains its activity after having been centrifuged at  $20,000 \times g$  for 2 hours.

## EXPERIMENTAL

### *Preparations*

*Keilin and Hartree Oxidase* (6)—Fresh lamb heart was minced by being passed once through a hand-operated meat grinder. It was washed with cold running tap water until the wash water was free or nearly free of hemoglobin. The washed tissue was then pressed in a bag made of cheese-cloth to remove excess water. To each 50 gm. of heart were added 150 ml. of ice-cold 0.04 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer, pH 7.0. The mixture was homogenized in a Waring blender for 2.5 minutes in the cold and then centrifuged at  $5^\circ$  for 15 minutes at 3500 R P M (approximately  $2500 \times g$ ). The cloudy supernatant was decanted into a beaker without special precautions (the precipitate was discarded) and treated with 25 ml. of ice-cold 0.2 M  $\text{CH}_3\text{COONa-CH}_3\text{COOH}$  buffer, pH 4.6. The insoluble oxidase complex was brought down by centrifugation at  $5^\circ$  for 10 minutes at 3500 R P M (approximately  $2500 \times g$ ). The supernatant was poured off without special precautions. The precipitate was weighed and suspended in an equal weight of 0.1 M  $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$  buffer, pH 7.4.

\* Aided by a grant from the John and Mary R. Markle Foundation.

Cytochrome *c* was prepared by the method of Keilin and Hartree (7) with the following modifications (1) after the precipitated cytochrome was washed with saturated  $(\text{NH}_4)_2\text{SO}_4$ , the red solid was dissolved in water and dialyzed overnight against water instead of against 1 per cent sodium chloride, (2) on the following day the solution was centrifuged for 5 minutes at 3500 R P M ( $2500 \times g$ ) in celluloid tubes and the supernatant lyophilized to yield approximately 150 mg of cytochrome *c* per kilo of beef heart (52 per cent pure)

*Test of Enzyme Activity*—The method used for testing the activity of the cytochrome oxidase complex was the one adapted by Haas (1) from the methods of Keilin and Hartree (6) and Stotz, Sidwell, and Hogness (8), except that buffer of pH 7.4 was used. The gas phase was air and the temperature  $25^\circ$ . Each Warburg vessel contained 3.0 mg of hydroquinone in 0.25 ml of water (side arm), 1.0 mg of cytochrome *c* in 0.5 ml of water, 0.25 or 0.5 ml of enzyme preparation or 0.16 per cent sodium desoxycholate, and 0.1 M  $\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$  buffer of pH 7.4 to make the total volume 3.0 ml. The upper limit of the oxygen uptake of this system is approximately 90 c mm in 15 minutes.

*Keilin and Hartree Oxidase*—When 1 ml of the final Keilin and Hartree (K and H) oxidase preparation was further diluted to 25 ml with water, the protein content was reduced to somewhat less than 1 mg per ml. The oxygen consumption was approximately 350 c mm per ml per hour when tested with the hydroquinone-cytochrome *c*-phosphate buffer system. The results of two typical experiments gave  $\text{QO}_2$  protein values of 400 and 370, respectively.

*Effect of Sodium Desoxycholate*—The first attempt to treat the Keilin and Hartree (K and H) oxidase with sodium desoxycholate yielded an active preparation. Centrifugation of the desoxycholate-treated homogenate (ground in a glass tissue grinder) for 1 hour at  $18,000 \times g$  yielded a supernatant which was clear and red-brown in color. When 0.25 ml of this concentrated supernatant was tested in the Warburg respirometer, the oxygen consumption was at a maximum. Therefore it was decided next to test the effect of dilution on the activity of the complex.

To 2 ml of the cold K and H preparation were added 80 mg of sodium desoxycholate and the mixture was ground intermittently in the cold room at  $4^\circ$  for 10 minutes. Following centrifugation at  $20,000 \times g$  for 1 hour, the clear supernatant was decanted and 1 ml was diluted to the desired volume with cold distilled water. The results presented in Table I indicate that optimum activity was reached at a dilution of 1:25, that this activity was maintained, or perhaps slightly improved, to a dilution of 1:100, and that thereafter the  $\text{QO}_2$  protein values declined. The dilution of 1:25 was chosen for the experiments that follow, since at this dilution a reasonably high, yet submaximum oxygen consumption was obtained.

*Varying Desoxycholate Concentration*—The first concentration of desoxycholate that was used, as reported above, was 4 per cent (40 mg per ml of K and H oxidase). In order, however, to conserve desoxycholate, it was decided to reduce the percentage to a minimum. Accordingly, five preparations were made by grinding the K and H oxidase in a mortar at 4° with various amounts of desoxycholate, namely, 5, 10, 20, 30, and 40 mg per ml of K and H oxidase. In the experiment reported in Fig 1 the preparations were centrifuged for 2 hours at  $20,000 \times g$ . All supernatants were decanted and diluted 1:25 with cold distilled water. The addition of 20 mg of desoxycholate per ml (2 per cent) gave a supernatant of low activity. When 30 mg of desoxycholate per ml were used (3 per cent), the  $QO_2$  protein was the expected  $1000 \pm$ . The activity was not further improved with

TABLE I  
*Effect of Dilution*

Oxidase dilution	Protein <i>mg per 0.25 ml</i>	15 min oxygen uptake <i>c mm per 0.25 ml</i>	$QO$ protein
1:10	0.515	78	550
1:25	0.173	49	970
1:75	0.058	24	1170
1:100	0.052	20	1000
1:150	0.029	12	630
1:200	0.022	8	180
Control, pH 7.4*		7	

\* 0.25 ml of 0.16 per cent sodium desoxycholate

4 per cent desoxycholate. From these results it appeared that, since 20 mg of desoxycholate per ml of K and H oxidase were dissolving a protein of low activity, the addition of more desoxycholate to the precipitate following centrifugation should yield a preparation of much higher activity.

*Partial Purification with Desoxycholate*—To 2 ml of cold K and H oxidase were added 40 mg of desoxycholate and the mixture was ground intermittently in a cold mortar at 4° for 10 minutes. Following centrifugation at  $18,000 \times g$  for 1 hour in the cold room (supernatant termed Fraction 0-2), the precipitate was ground for 5 minutes in a mortar with a cold solution of 4 per cent sodium desoxycholate in 0.1 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer of pH 7.4. The suspension was centrifuged at  $18,000 \times g$  for 1 hour and the supernatant decanted and termed Fraction 2-4. All supernatants were diluted 1:25 with cold distilled water as soon as possible after having been decanted. The results demonstrated a partial purification of those components of the oxidase complex that mediate the oxidation of hydroquinone in the presence of cytochrome c. The protein of Fraction 0-2 was high in

relation to the oxygen consumption, indicating that it consists in large measure of proteins that are inactive. Fraction 2-4 had the remainder of the proteins that were usually dissolved by 4 per cent desoxycholate and they were proteins of high activity ( $QO_2$  protein, 2480). The supernatant of Fraction 2-4 was clear and yellow in color.

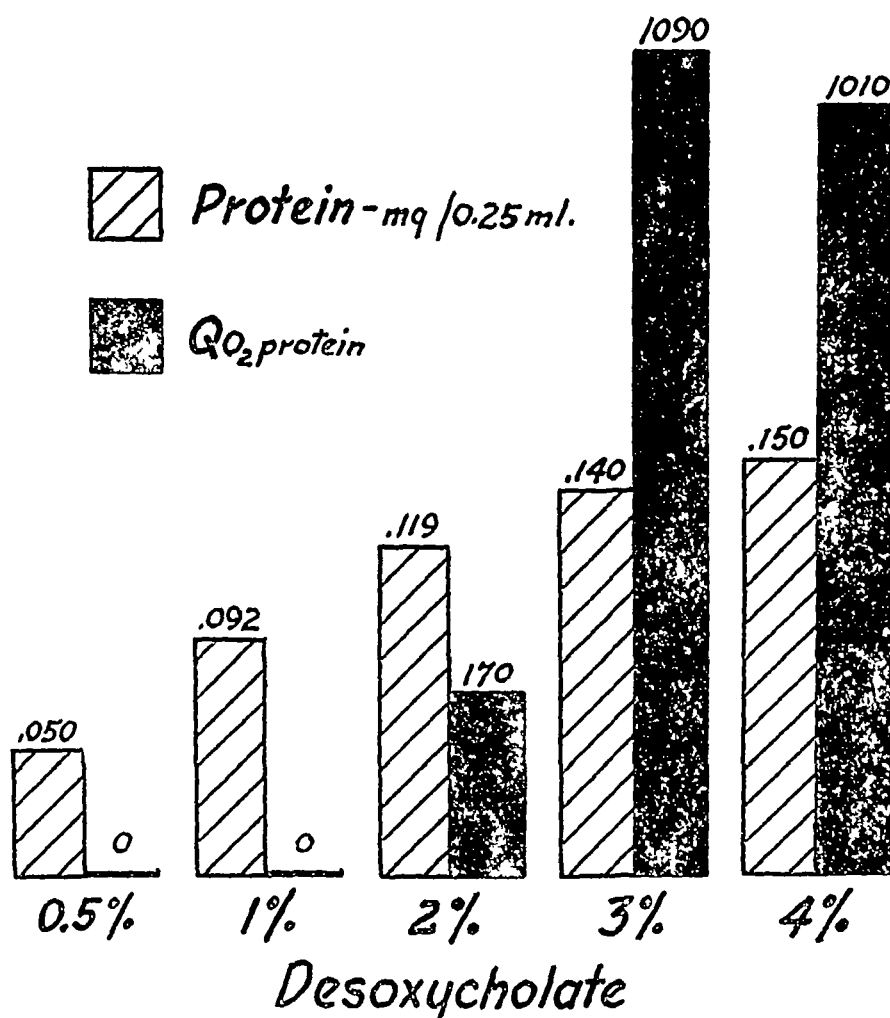


FIG 1 Effect of various concentrations of sodium desoxycholate

The importance of the desoxycholate-protein ratio is borne out by a consideration of the experiment reported in Table II. The addition of 2 per cent of desoxycholate dissolved much protein of low activity, whereas the addition of 2.5 per cent of desoxycholate to another sample dissolved only a little more protein and considerably increased the oxygen consumption. If the increase in oxygen consumption could be attributed to the extra 0.055

mg of protein per 0.5 ml, then the  $QO_2$  protein for that fraction (No. 2-2.5) could be calculated to be more than 2000. This value is of the same order as that obtained with Fraction 2-3 (2 per cent desoxycholate followed by 3 per cent desoxycholate in 0.1 M  $Na_2HPO_4$ - $KH_2PO_4$  buffer, pH 7.4, to the precipitate). Fraction 2.5-4 and Fraction 3-4 (preceded by Fraction 0-3 not tested) show that further increases in the amount of desoxycholate added cause proteins of low activity to be dissolved again.

*Effect of Sodium Cyanide and Sodium Azide*—The effect of sodium cyanide and sodium azide was tested at one concentration of each inhibitor. The enzyme solution was a 3 per cent desoxycholate-K and H oxidase that had been centrifuged for 1 hour at  $20,000 \times g$  and diluted 1:25 with cold distilled water. Inhibition was complete with both substances at a final concentration of  $1 \times 10^{-3}$  M, indicating that cytochrome oxidase is one of the enzymes in the desoxycholate preparation.

TABLE II  
Partial Separation with Sodium Desoxycholate

Desoxycholate Fraction No.	Protein mg per 0.5 ml	15 min. oxygen uptake c.mm. per 0.5 ml	$QO_2$ protein
0-2	0.333	9	40
0-2.5	0.388	40	370
2-3	0.104	56	1920
2.5-4	0.087	40	1650
3-4	0.056	19	930
Control, pH 7.4*		6	

\* 0.25 ml of 0.16 per cent sodium desoxycholate

*Effect of Carbon Monoxide*—The enzyme preparation was a 4 per cent desoxycholate-K and H oxidase that had been passed through a Seitz filter. The clear red-brown filtrate was lyophilized from the frozen state to yield a stable preparation. The dry preparation ( $QO_2$  protein 1100) which contained sodium desoxycholate and sodium and potassium acid phosphates in addition to the proteins was dissolved in distilled water for use. Carbon monoxide caused a 68 per cent inhibition during the first 15 minutes in the absence of light and the inhibition was completely reversed when the light of a 500 watt projection lamp was allowed to fall on the vessels.

*Oxidation of Reduced Cytochrome c*—The enzyme preparation was the same lyophilized preparation that was used in the previous experiment for the demonstration of carbon monoxide inhibition. The cytochrome c solution was reduced with sodium hydrosulfite and the excess hydrosulfite oxidized by aeration. The rate of the oxidation of the reduced cytochrome c was



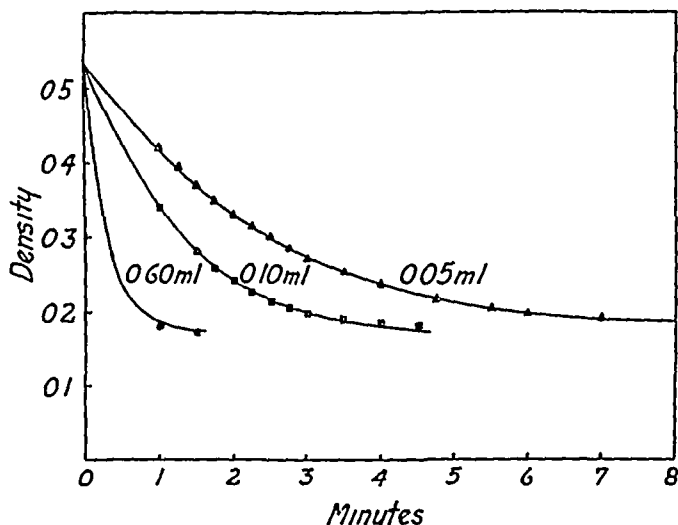


FIG 2 Change in density ( $\log I_0/I$ ) of reduced cytochrome *c* solution with time. Oxidase preparation 0.378 mg of protein per ml. The spectrophotometer cell contained 2 mg of cytochrome *c* in 1.0 ml of distilled water, 0.4 ml of 0.1 M  $\text{KH}_2\text{PO}_4$ - $\text{Na}_2\text{HPO}_4$  buffer, pH 7.1, and 2.0 ml of distilled water. The volume was brought to 4.0 ml by the addition of the enzyme solution (0.60 ml) or of the enzyme solution (0.10 or 0.05 ml) plus distilled water (0.50 or 0.55 ml).

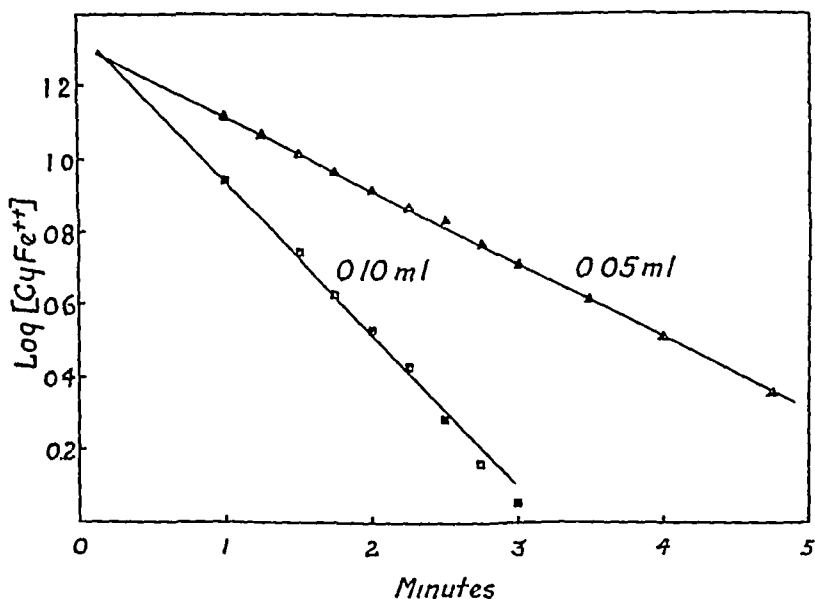


FIG 3 Rate of oxidation of reduced cytochrome *c*.

followed with a Beckman spectrophotometer<sup>1</sup> at 550 m $\mu$  (slit width 0.02 mm) when 0.60, 0.10, or 0.05 ml of the enzyme solution was added. The

<sup>1</sup> Made available through the courtesy of Dr. H. G. Albaum.

results are presented in Fig 2, where the density ( $\log I_0/I$ ) of the solution of reduced cytochrome *c* is plotted against the time in minutes. From the data obtained with 0.10 and 0.05 ml of the enzyme solution it can be calculated that the log of the concentration of reduced cytochrome *c* ( $\log \text{CyFe}^{++}$ ), when plotted against time in minutes, yields a straight line (Fig 3). The calculations are made in accord with those reported by Haas, Horecker, and Hogness (9) for the reduction of oxidized cytochrome *c* by cytochrome *c* reductase of yeast. The slope of the line obtained with 0.10 ml of the enzyme is almost exactly 2 times that obtained with 0.05 ml, indicating that the rate of oxidation is proportional to the enzyme concentration.

#### DISCUSSION

The experiments that have just been discussed were preceded by many attempts to improve the activity of the Keilin and Hartree preparation by physical means. Repeated failures prompted a search for chemical agents to dissolve the enzymes. It seemed possible that the complex might be a lipoprotein, and fat emulsifiers were, therefore, considered as active agents. Immediately thereafter, the work of Hopkins, Lutwak-Mann, and Morgan (3) was found, and bile salts, in view of their surface active properties, seemed a reasonable choice. The idea that the complex might be a lipoprotein has not been discarded.

The constituent enzymes of this complex that oxidizes hydroquinone in the presence of added cytochrome *c* have been only superficially investigated in this work. Added cytochrome *c* is necessary to catalyze the uptake of oxygen. The presence of cytochrome oxidase is indicated by the action of the three inhibitors, sodium cyanide, sodium azide, and carbon monoxide. Unfortunately, there are no such inhibitors for the identification of cytochrome *a*.

The use of a buffer of pH 7.4 in the test for enzyme activity, instead of one of pH 7.1, was a somewhat unfortunate choice, in view of the relatively large blank that occurs with the pH 7.4 buffer. However, when comparative studies were made at pH 7.4 and 7.1, the  $\text{QO}_2$  protein values were the same.

#### SUMMARY

1. A soluble and active cytochrome oxidase has been prepared by adding sodium desoxycholate to a Keilin and Hartree (K and H) oxidase preparation from lamb heart. When tested with the hydroquinone-cytochrome *c* system at pH 7.4, the enzymes are found in the supernatant after centrifugation at  $20,000 \times g$  for 2 hours.

2. When 3 or 4 per cent of desoxycholate is added to the K and H oxidase,

the supernatant, after high speed centrifugation and dilution, is approximately 2.5 times as active as the original, dilute K and H preparation

3 A partial purification of the oxidase can be effected by adding a solution of desoxycholate to the precipitate of a desoxycholate-treated K and H oxidase and by discarding the residue that remains. Separation of the precipitate can be accomplished by either high speed centrifugation or by filtration through a Sartz filter. The  $QO_2$  protein obtained by such a procedure is approximately 2000 (5 times as active as the K and H oxidase)

4 Sodium cyanide, sodium azide, and carbon monoxide in the absence of light inhibit the oxidase contained in a desoxycholate preparation of the K and H oxidase. A reversal of the carbon monoxide inhibition is obtained in light

5 The oxidation of reduced cytochrome *c* has been demonstrated spectrophotometrically

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A NEW SYNTHESIS OF S-( $\beta$ -AMINO- $\beta$ -CARBOXYETHYL)HOMO-  
CYSTEINE (CYSTATHIONINE) AND S-BIS( $\gamma$ -AMINO-  
 $\gamma$ -CARBOXYPROPYL)SULFIDE  
(HOMOLANTHIONINE)\*

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Cystathionine has been synthesized by Brown and du Vigneaud (1) by condensing homocysteine with  $\alpha$ -aminochloropropionate in an alkaline aqueous medium

Snyder *et al* (2, 3) have shown that 3,6-bis( $\beta$ -chloroethyl)2,5-diketopiperazine is a useful intermediate in the synthesis of methionine and other thio ethers. The synthesis of cystathionine and homolanthionine described here is based on the condensation of cysteine or homocysteine with the dichloroethyldiketopiperazine of Snyder *et al* (2) in liquid ammonia, followed by the acid hydrolysis of the condensation products to yield cystathionine and homolanthionine respectively. These reactions are represented in the accompanying diagram

As far as we are aware, homolanthionine has not been prepared previously. The yields of cystathionine obtained by this method are considerably higher than those reported previously (1). Homolanthionine and the intermediates in the synthesis of homolanthionine and cystathionine (the corresponding diketopiperazine derivatives) are of some physiological interest from the standpoint of their availability to animals for growth purposes in lieu of methionine or cystine. Enzymatic cleavage of these thio ethers *in vitro* also suggests interesting possibilities. Such studies are at present in progress.

By a suitable choice of the isomers of cystine and homocysteine (or methionine) and of the dichloroethyldiketopiperazine or of  $\gamma$ -chloro- $\alpha$ -aminobutyric acid it ought to be possible to prepare any of the isomers of cystathionine and homolanthionine in a similar manner<sup>1</sup>. We are investigating this possibility.

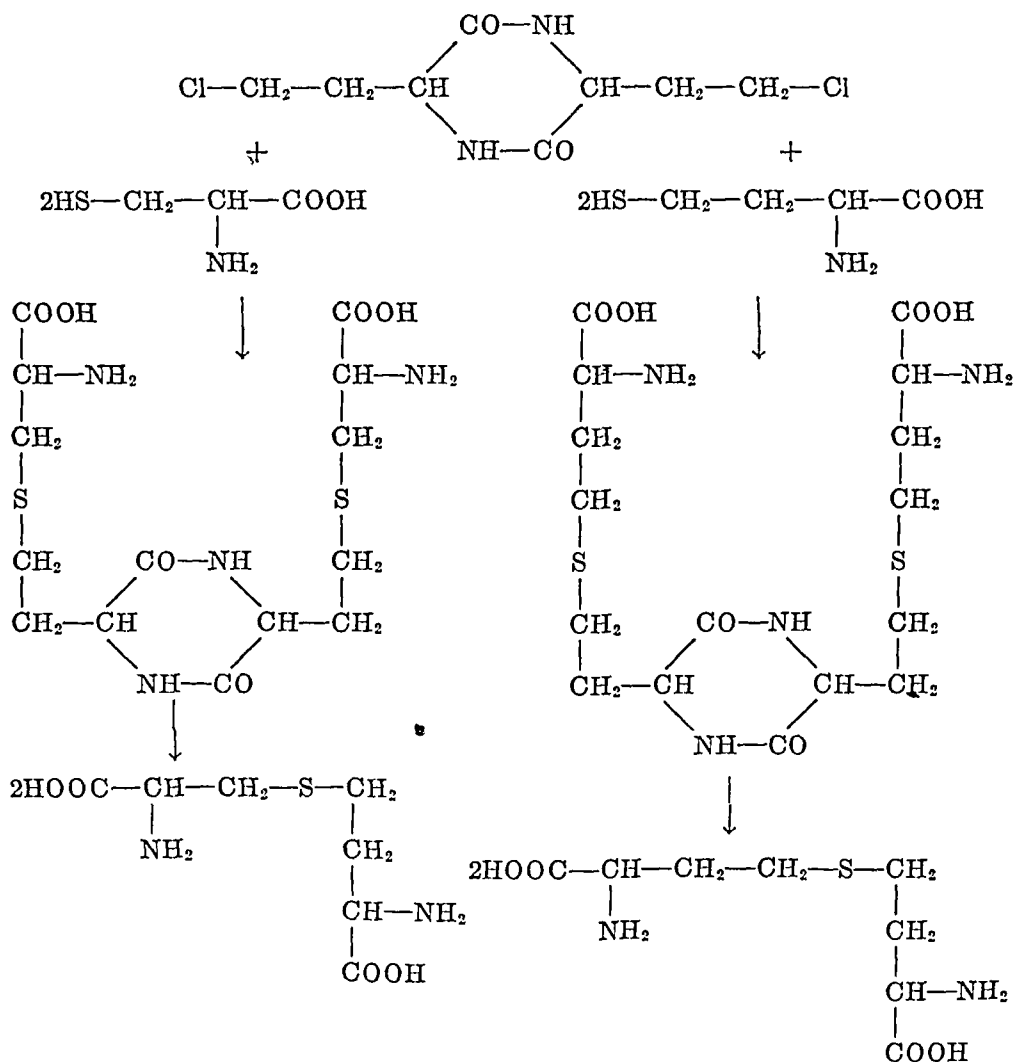
#### EXPERIMENTAL

*S*,6-Bis(*S*-( $\beta$ -amino- $\beta$ -carboxyethyl)ethyl)2,5-diketopiperazine—10 gm of metallic sodium were dissolved in about 500 ml of liquid ammonia and 24 gm

\* Aided by an anonymous grant in memory of Susanna Dercum

<sup>1</sup>  $\gamma$ -Chloro  $\alpha$ -aminobutyric acid can be prepared from  $\gamma$ -hydroxy- $\alpha$ -aminobutyric acid (homoserine). The latter has been resolved by Armstrong (4).

of L-cystine were added to the solution in small portions with constant agitation of the reaction mixture (5). The blue color of the excess sodium was discharged by a few crystals of L-cystine. 24 gm of 3,6-bis( $\beta$ -chloroethyl)-2,5-diketopiperazine (synthesized according to Snyder *et al* (2)) were added to the reaction vessel in small portions with thorough mixing of the



solution. After all of the diketopiperazine derivative had been added, ammonia was allowed to evaporate, and the reaction vessel was evacuated in order to remove as much ammonia as possible from the residue. The latter was then dissolved in about 300 ml of ice-cold water and the solution was filtered through a bed of nonit. The filtrate was adjusted with hydrochloric acid to about pH 9, and about 3 gm of sodium cyanide were added to the solution in order to convert any unchanged cystine to the soluble

form (6) After 10 to 15 minutes the pH of the solution was adjusted with hydrochloric acid to 6.0 (under a hood) After standing in the refrigerator overnight, the precipitated material was removed by filtration, washed with cold water, and then recrystallized from dilute ammoniacal solution by addition of hydrochloric acid to pH 6 The crystallized product was suspended in water, removed by filtration, and washed successively with water, ethanol, and ether After drying *in vacuo* at 100° over  $P_2O_5$ , the product decomposed at 260–265°<sup>2</sup> The yield was 31 gm, or 76 per cent of the theoretical amount The analysis of the material gave the following results

$C_{11}H_{14}N_4S_2O_6$	Calculated	C 41.18, H 5.88, N 13.51, S 15.63
	Found	" 40.60, " 6.14, " 13.44, " 15.45

The compound gave a negative sodium cyanide-nitroprusside test The ninhydrin test was positive

*Cystathionine*—10 gm of the diketopiperazine derivative prepared as described above were refluxed with 150 ml of 20 per cent hydrochloric acid for 3 hours The solution was then evaporated *in vacuo* to dryness on a steam bath, and the residue was dissolved in 50 ml of water As a rule, only a slight darkening of the solution of the diketopiperazine derivative in hydrochloric acid resulted after 3 hours of refluxing The solution was decolorized and filtered The filtrate was adjusted with dilute sodium or ammonium hydroxide to pH 6, and cooled in an ice bath Crystallization can be speeded up by the addition of ethanol (to about 40 per cent concentration with respect to ethanol) After standing in the refrigerator overnight, the crystallized material was removed by filtration and washed with cold water After recrystallization from dilute ethanol and drying *in vacuo* at 100° over  $P_2O_5$ , the product weighed 10 gm, or 90 per cent of the theoretical amount It decomposed at 270–274° The sodium cyanide-nitroprusside test on the material was negative The analytical results are shown below

$C_4H_8N_2SO_3$	Calculated	C 37.38, H 6.36, N 12.61, S 14.41
	Found	" 37.01, " 6.47, " 12.60, " 14.18

Since L-cystine and racemic 3,6-bis( $\beta$ -chloroethyl)2,5-diketopiperazine were used in the present synthesis, the resulting product is probably a mixture of L-cystathionine and L-allo-cystathionine The possibility of separation of the two stereoisomers by fractional crystallization from water or other solvents is being investigated at present<sup>3</sup>

<sup>2</sup> All melting points were made in open capillary tubes and are uncorrected

<sup>3</sup> The mixture of isomers of cystathionine prepared by this method is biologically equal to L-cystine or DL-methionine when fed to rats maintained on a low casein diet This suggests that no appreciable racemization of the cysteine moiety of cysta-

The dibenzoyl derivative of the cystathionine synthesized as described above was prepared in the usual manner. After recrystallization from 70 per cent ethanol and drying *in vacuo* at room temperature over  $P_2O_5$ , it had the following composition

$C_{21}H_{22}N_2SO_6$	Calculated	C 58.61, H 5.11, N 6.51, S 7.45
	Found	" 58.83, " 5.42, " 6.47, " 7.64

*3,6-Bis(S-( $\gamma$ -amino- $\gamma$ -carboxypropyl)ethyl)2,5-diketopiperazine* From *Methionine*—We have reported the synthesis of S-benzylhomocysteine by condensing benzyl chloride with the reduction product of methionine with sodium in liquid ammonia (7). By a similar procedure, S-carboxymethylhomocysteine and S-carboxyethylhomocysteine were also prepared from methionine and chloroacetic acid and  $\beta$ -bromopropionic acid respectively (8). In the present synthesis of homolanthionine, the dichloroethyl-diketopiperazine was condensed with the reduction product of methionine with sodium in liquid ammonia by a procedure similar to that described above for cystathionine. From 10 gm of DL-methionine and 8 gm of 3,6-bis( $\beta$ -chloroethyl)2,5-diketopiperazine, 7.5 gm of 3,6-bis(S-( $\gamma$ -amino- $\gamma$ -carboxypropyl)ethyl)2,5-diketopiperazine were obtained. After recrystallization from dilute ethanol and drying *in vacuo* at  $100^\circ$ , the material decomposed at  $270$ – $273^\circ$  and had the following composition

$C_{16}H_{24}N_4S_2O_6$	Calculated	C 44.04, H 6.42, N 12.84, S 14.68
	Found	" 43.69, " 6.61, " 12.64, " 14.33

*From Homocystine*—Homocystine was prepared according to Snyder *et al.* (3). By the procedure described above, 18 gm of 3,6-bis(S-( $\gamma$ -amino- $\gamma$ -carboxypropyl)ethyl)2,5-diketopiperazine were obtained from 13.2 gm of homocystine and 12.05 gm of dichloroethyldiketopiperazine. The product had the same decomposition range as that shown by the product obtained from methionine, and had the following composition

$C_{16}H_{24}N_4S_2O_6$	Calculated	C 44.04, H 6.42, N 12.84, S 14.68
	Found	" 43.80, " 6.57, " 12.60, " 14.40

Both products gave a negative sodium cyanide-nitroprusside test. The ninhydrin test was positive.

*Homolanthionine*—10 gm of the above diketopiperazine derivative were hydrolyzed in 150 ml of 20 per cent hydrochloric acid in the manner described above for the cystathionine derivative. The yield of homo-

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thionine occurred during the synthesis of cystathionine, and that the mixture probably consists of L-cystathionine and L-allo-cystathionine (Stekol, unpublished data).

lanthionine was 9.3 gm. After drying *in vacuo* at 100° over  $P_2O_5$ , it decomposed at 269–272°. The analytical results are shown below

$C_5H_{12}N_2SO_4$	Calculated	C 40.68, H 6.78, N 11.86, S 13.56
	Found	" 40.10, " 6.76, " 12.27, " 13.63

Since in the above synthesis racemic methionine, homocystine, and dichloroethyldiketopiperazine were used, the resulting product is probably a mixture of racemic and meso forms of homolanthionine. The possibility of the separation of the isomers by fractional crystallization from water and other solvents is being investigated at present.

S-Benzylhomocystine can also be used instead of either methionine or homocystine in the preparation of homolanthionine by the procedure described above.

#### SUMMARY

Cystathionine and homolanthionine have been prepared in good yields by the condensation of cysteine or homocystine with 3,6-bis( $\beta$ -chloroethyl)-2,5-diketopiperazine in liquid ammonia, followed by acid hydrolysis of the condensation products.

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# A NEW METHOD FOR THE DETERMINATION OF THE ENZYME ALDOLASE

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At the present time the term aldolase is usually applied to the enzyme which catalyzes the conversion of 1 molecule of fructose diphosphate to 2 molecules of triose phosphate, although the name zymohevas was used formerly, and still is used frequently. Aldolase has been prepared in crystalline form from muscle by Warburg and Christian (1) and by Taylor, Green, and Cori (2).

Because of the rôle of aldolase in the glycolysis scheme, it is of interest to determine this enzyme in glycolyzing tissues, particularly in tumors. An increase in the aldolase content of the plasma of tumor-bearing animals as compared with normal animals has been reported by Warburg, and this fact alone would seem to make it desirable to establish a method for determining aldolase which is simpler than the complicated method employed by Warburg and Christian. The method of determining aldolase used by Herbert *et al* (3), which involves a determination of an increase in alkali-labile phosphate, does not appear to be well suited to the determination of aldolase in the presence of considerable amounts of inorganic or labile phosphate such as occur in tissues and plasma. The presence of competing enzyme systems may upset results both by the method of Warburg and Christian (1) and of Herbert *et al* (3).

We wish to report a simple method for determining aldolase which is applicable to all tissues thus far studied, including liver, kidney, spleen, muscle, plasma, tumor tissue, and isolated cell nuclei from liver, kidney, and pancreas, as well as to solutions of crystallized aldolase. The method no doubt could be made more precise by a considerable amount of extra effort, but it appears to be satisfactory in its present state in that it yields results well within the differences in aldolase activities encountered in studying the various tissues listed above, and in that it can be used to obtain useful kinetic studies of the enzyme.

## Outline of Method

The method involves a determination of the triose phosphate formed by the action of aldolase on the fructose diphosphate which is employed as substrate. The triose phosphate is converted by the action of hot con-

centiated sulfonic acid to acetaldehyde, probably by way of methylglyoxal as intermediate, and the acetaldehyde is then measured by color formation with *p*-hydroxydiphenyl, as in the Barker-Summerson method for lactic acid (4). In fact the determination of the triose phosphate is a direct application of the Barker-Summerson method for lactic acid, except that the step of purification with copper sulfate and calcium hydroxide, which removes some triose phosphate, is omitted. The fructose diphosphate, which must be quite pure, causes a light yellow color which, however, is compensated for by running a blank determination and by using the proper filter.

Apparently both dihydroxyacetone phosphate and 3-phosphoglycer-aldehyde yield acetaldehyde in the determination in roughly comparable amounts, so that the presence of isomerase should not appreciably affect the results of the determination.

#### *Outline of Studies of Kinetics of Aldolase Carried Out by Use of New Method*

Our kinetic studies have been of a practical nature, designed principally to establish optimal conditions for determining aldolase. We have investigated the effect of substrate concentration on reaction velocity, the time course of the reaction, the effect of enzyme concentration on the reaction velocity, the effect of pH on the reaction velocity, the effect of temperature on reaction velocity, and the turnover number, or moles of substrate decomposed per mole of enzyme per minute, under standard conditions. The effect of copper on the enzyme also has been studied.

#### EXPERIMENTAL

##### *Determination of Aldolase*

Place 0.7 ml. of 0.1 M disodium fructose diphosphate<sup>1</sup> adjusted to pH 7.0 in a 15 ml. centrifuge tube. If tissue or nuclei are to be analyzed, add

<sup>1</sup> Commercial fructose diphosphate can be used if it is purified as follows. Dissolve 10 gm. of barium fructose diphosphate in 200 ml. of distilled water to which is added dropwise sufficient glacial acetic acid to give a nearly clear solution at pH 3.0 to 3.5. The slight amount of residue which remains is then filtered off. Now neutralize the solution by adding 10 per cent NaOH and finally 1 N NaOH until the pH is between 7.0 and 7.5. Isolate the precipitate of barium fructose diphosphate by centrifugation. Wash once with water, once with 25 per cent alcohol, once with 50 per cent alcohol, twice with 95 per cent alcohol, and twice with ether. Dry at room temperature and then place in a desiccator. To convert the barium salt to the sodium salt, dissolve 1.37 gm. of the purified barium salt in 15 ml. of 1 N HCl to yield a nearly clear solution of the barium salt. Now add 5 ml. of molar Na<sub>2</sub>SO<sub>4</sub> solution slowly and with stirring. Centrifuge off the barium sulfate and test the supernatant for completeness of precipitation by adding an extra drop of sodium sulfate. If

0.1 ml of 0.02 per cent iodoacetic acid previously neutralized to pH 7.0 with NaOH in order to block the action of 3-phosphoglyceraldehyde dehydrogenase. Next add 0.2 ml of properly diluted enzyme solution or of homogenized tissue suspension,<sup>2</sup> plasma, or of isolated tissue cell nuclei and start timing the reaction at this point. If pure enzyme is being analyzed, 0.1 ml of water can be added in place of the 0.1 ml of iodoacetate if desired. Now incubate the reaction mixture at 25° for 15 minutes.

Stop the reaction at the end of 15 minutes by adding 4 ml of 8 per cent trichloroacetic acid. Centrifuge the precipitated protein, a procedure which usually requires 5 minutes. Now pipette 1 ml of the supernatant solution into a large Pyrex test-tube, place the tube in an ice-water bath, and add slowly and with stirring 6 ml of concentrated H<sub>2</sub>SO<sub>4</sub> from a fine tipped burette. Avoid local overheating by too rapid addition of the acid.

Place the tube containing the deproteinized reaction mixture and acid in a bath of boiling water and allow it to remain there for 3 minutes. Next cool the tube under tap water until the contents are at approximately room temperature and add 1 drop of a 4 per cent solution of cupric sulfate and 2 drops of the *p*-hydroxydiphenyl reagent made up according to the directions of Barker and Summerson (4).<sup>3</sup> (Use the same fine tipped medicine droppers for all experiments.)

Now incubate the test-tube at 27–30° for 30 minutes and then place in a bath of boiling water for 60 to 90 seconds. Cool the tube under tap water until the contents have reached room temperature approximately, and transfer the contents to a colorimeter tube, avoiding condensed moisture

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precipitation is complete, adjust the pH to about 7.2 with NaOH solution, using a 10 per cent solution of NaOH at first and finally 1 N NaOH for the final adjustment. Filter the solution and dilute to 25 ml. This gives a solution of approximately 0.1 M fructose diphosphate as the sodium salt. Keep in the ice box for not more than 1 week.

<sup>2</sup> We have employed freshly prepared tissue homogenates for all analyses on tissues, using a ground glass homogenizer cooled in a bath of ice and water for grinding the tissue. Distilled water has been used for medium. For example, in the case of liver, approximately 1 gm of liver (wet weight) was homogenized with 5 ml of distilled water until practically cell free. The resulting suspension was diluted with sufficient distilled water to give a final suspension of about 10 mg (dry weight) per ml. In the case of muscle, the original homogenate was made by grinding 0.25 gm of muscle (wet weight) with 5 ml of distilled water. This suspension should then be diluted at least 1:4 with distilled water before use if 0.2 ml portions are to be taken for analysis. Homogenates of other tissues are prepared in a similar manner. The final dilution should be adjusted so that the colorimeter reading after incubation is at least 100 above the blank reading. Serum should not be diluted at all if 0.2 ml portions are being used for analysis.

<sup>3</sup> 1.5 gm of *p*-hydroxydiphenyl (Eastman grade) are dissolved in 5 per cent NaOH, and the solution is then diluted 1:10 with distilled water. The diluted solution keeps for long periods at room temperature in a colored stoppered bottle.

at the top of the test-tube. Read the color value immediately in a Klett-Summeison colorimeter, using Filter 56, or in another suitable colorimeter.

A blank determination must be made at the same time the enzyme is being determined. This is done by adding to the 15 ml. centrifuge tube first the enzyme solution, then the trichloroacetic acid, then the iodoacetate, and finally the fructose diphosphate. The blank is then treated exactly as described above. The reading of the blank, which usually varies from 125 to 250 in the Klett-Summeison instrument, depending upon what tissue is employed, is subtracted from the reading for the enzyme determination, which should be in the neighborhood of 300 to 500 for best results.

A considerable portion of the blank value comes from the yellow color produced by the action of the sulfuric acid on the fructose diphosphate.<sup>4</sup> When blood plasma or whole tissues are employed, relatively high blank values are observed, which probably are caused by some precursor of acetaldehyde, particularly lactate. Isolated cell nuclei yield considerably lower blank values.

In carrying out determinations of aldolase, the same precautions must be observed that are mentioned by Barker and Summeison (4) for the determination of lactic acid. For instance, great care must be taken to avoid contaminating the contents of the tubes with lactate from the fingers, and all time intervals should be carefully measured.

#### *Experiments with Triose Phosphates*

*Dihydroxyacetone Phosphate*—This triose phosphate probably can best be prepared by the low temperature phosphorylation of dihydroxyacetone by the method of Kiessling (5). The dihydroxyacetone is obtainable by a method involving the use of bacteria (6). However, these procedures are time-consuming and we attempted to obtain crude dihydroxyacetone phosphate directly by bromine water oxidation of  $\alpha$ -glyceryl phosphate by a modification of the procedure reported by Bailly (7). Our  $\alpha$ -glyceryl phosphate was prepared by heating a solid mixture of  $\alpha$ - and  $\beta$ -glyceryl phosphates with 5 per cent HCl, which brings about an equilibration resulting in a mixture of 95 per cent of the  $\alpha$  and 5 per cent of the  $\beta$  forms. The 95 per cent  $\alpha$ -glyceryl phosphate was then isolated as the barium salt. In most of the experiments we treated the barium salt of  $\alpha$ -glyceryl phosphate with bromine water, but the sodium salt also was tried, exactly as described by Bailly (7).

<sup>4</sup> Some commercial fructose diphosphate samples contain much heavy metal which must be removed by treatment with  $H_2S$ . Other impurities often occur which cause an excessively high color in the blank determination and which lower aldolase activity. This lowered activity can be partially restored by adding cysteine (Stotz *et al.*, this department). Cysteine has little or no effect on aldolase activity if sufficiently pure substrate is used.

In one experiment a 25 per cent aqueous solution of the barium salt of  $\alpha$ -glyceryl phosphate was prepared by adding sufficient 6 N HCl to the suspension of the glyceryl phosphate in water to lower the pH to 6.0. To the resulting clear solution the theoretical amount of bromine was added in small amounts, and 10 per cent NaOH was added as needed to keep the pH between 5.5 and 6.0. A precipitate developed during the reaction which contained some barium phosphate and apparently some of the desired product or its dimer.

From this reaction mixture a product was obtained as a barium salt which was judged to be not more than 15 per cent pure, according to an analysis for easily hydrolyzable phosphate. This product gave a strong resorcinol- $\text{H}_2\text{SO}_4$  test as reported by Bailly, and it also gave an intense color test with the Barker-Summerson reagent, although  $\alpha$ -glyceryl phosphate itself gives no color whatsoever. Since the most probable oxidation products in the reaction described above are dihydroxyacetone phosphate and dihydroxyacetone itself, rather than 3-phosphoglyceraldehyde or glyceraldehyde, it is very likely that dihydroxyacetone phosphate contributes strongly to the color test in our aldolase determination.

The method used by Bailly for preparing dihydroxyacetone phosphate, as well as simple modifications of this method, thus yielded only a very crude product which we were unable to purify appreciably. Judging from the results of some experiments carried out by us in an attempt to prepare the phenylhydrazone of dihydroxyacetone phosphate, and from a consideration of the probable high solubility of such a phenylhydrazone in water, it seems unlikely that the phenylhydrazone obtained by Bailly from his dihydroxyacetone phosphate preparation was in fact the phenylhydrazone of the latter compound, in spite of the apparently correct analytical figures which were reported.

*Calcium Salt of 3-Phosphoglyceraldehyde*—This material was prepared directly from the dioxane addition product of dimeric glyceraldehyde 1-bromide 3-phosphoric acid according to the directions of Baer and Fischer (8).<sup>5</sup> The calcium salt of 3-phosphoglyceraldehyde was found to give an intense color with the Barker-Summerson reagent. The color production per mole of the calcium salt of 3-phosphoglyceraldehyde was approximately 67 per cent of the color which was calculated to be produced from 1 mole of 3-phosphoglyceraldehyde plus 1 mole of dihydroxyacetone phosphate obtained by the action of aldolase on fructose diphosphate. (Total triose phosphate was measured by alkali-labile phosphate as described under the work on turnover number. 0.1 mg. of the calcium salt

<sup>5</sup> The dioxane addition compound was very generously supplied to us by Dr Baer and Dr Fischer. We gratefully acknowledge this gift, since it spared us much time and the difficulty of carrying out a sequence of unfamiliar reactions.

of 3-phosphoglyceraldehyde produced a color reading of 310 on the one-fifth aliquot used for the Barker-Summeison reagent )

### Kinetics of Aldolase As Measured by Our Method of Determination

**Reaction Velocity versus Substrate Concentration**—The effect of substrate concentration on reaction velocity is shown in Fig 1 The reaction velocity is measured as the colorimeter reading given by the aliquot with a reaction time of 15 minutes Such a small fraction of the substrate is decomposed that the reaction velocity can be considered as approximately constant over a 15 minute time interval, and hence the colorimeter reading

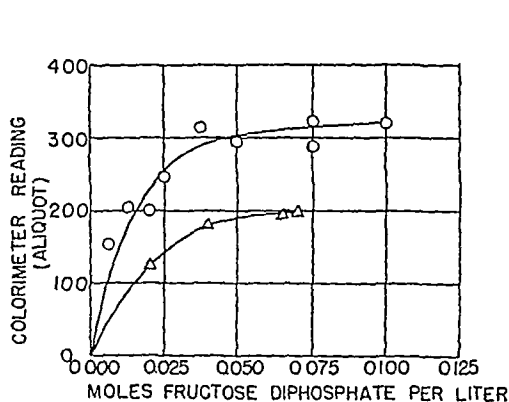


FIG 1

FIG 1 Effect of substrate concentration on aldolase activity at 25°, pH 7.2 Klett-Summerson colorimeter, Filter 56 O, crystallized aldolase, Δ, liver nuclei of rat

FIG 2 Lineweaver-Burk transformation for determining  $K_m$   $[S]/v = [S]/V + K_m/V$ . Data obtained from Fig 1 O, crystallized aldolase ( $K_m = 0.0086$ ), Δ, liver nuclei of rat ( $K_m = 0.021$ )

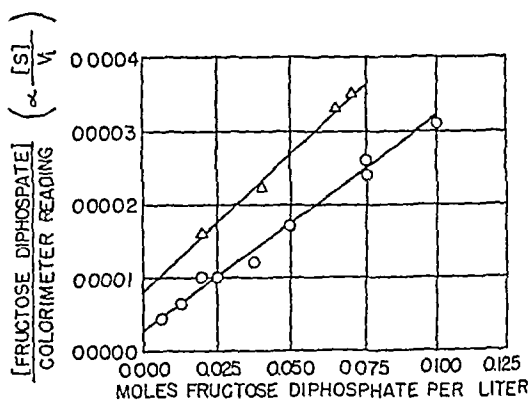


FIG 2

can be considered as being proportional to initial reaction velocity  $v$ . In Fig 1 values are plotted for pure enzyme and for isolated cell nuclei

In Fig 2 the data from Fig 1 are replotted in linear form by one of the improved transformations of Lineweaver and Burk (9) (indicated in Fig 2) From this linear plot the Michaelis-Menten constant for the enzyme  $K_m$  (10) is calculated This turns out to be about 0.009 for crystalline aldolase For the nuclei the value is 0.02 Although the order of magnitude of the two results is similar, the discrepancy shows that measurements carried out on nuclei differ in some way quantitatively from measurements carried out on the pure enzyme Why this should be so is not yet known

The Michaelis-Menten constant for aldolase was reported by Herbert *et al* (3) to be less than 0.001, but the decimal point in this figure appears to be misplaced We obtain a value of 0.0023 by treating the data as we

have treated ours, and therefore presume that these authors meant to state that the  $K_m$  value was less than 0.01

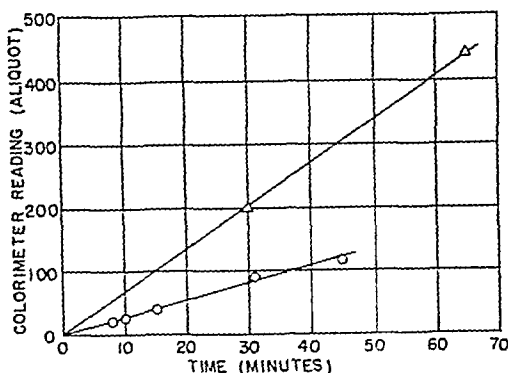


FIG 3

FIG 3 Effect of time on decomposition of fructose diphosphate by crystallized aldolase Klett-Summerson colorimeter, Filter 56 O, Δ, different dilutions of enzyme

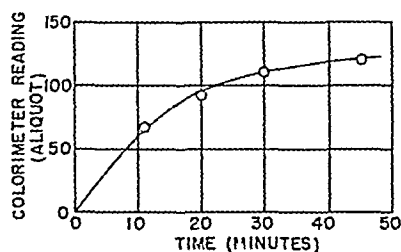


FIG 4

FIG 4 Effect of time on decomposition of fructose diphosphate by aldolase of isolated cell nuclei (rat liver) Klett-Summerson colorimeter, Filter 56

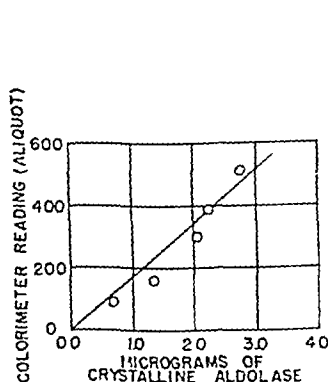


FIG 5

FIG 5 Colorimeter readings plotted against micrograms of crystallized aldolase Klett-Summerson colorimeter, Filter 56

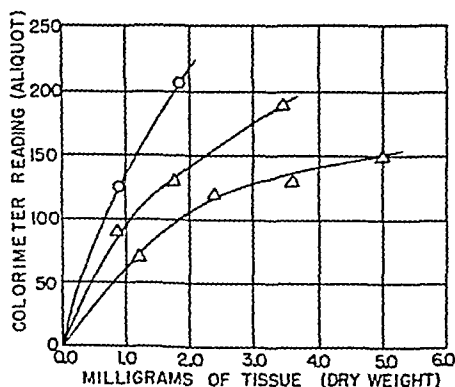


FIG 6

FIG 6 Effect of amount of material on colorimeter reading in aldolase determination for liver homogenate and cell nuclei of rat liver Klett-Summerson colorimeter, Filter 56 O, rat liver homogenate, Δ, liver nuclei of rat

*Time Relationships*—The results of plotting the colorimeter reading against time are shown for crystalline aldolase and liver cell nuclei in Figs 3 and 4. It can be seen that the reaction velocity is constant for a long



period with crystalline aldolase, but that it is constant for 15 minutes at the most with isolated cell nuclei. What causes a decrease in velocity in the case of the nuclei is not yet known. In these experiments optimal substrate concentration was used (0.07 M fructose diphosphate). The reaction should be of zero order theoretically under these conditions. It is of zero order for the crystalline enzyme, but it is not of zero order for the nuclei if time intervals greater than 15 minutes are employed.

*Reaction Velocity versus Amount of Enzyme Employed*—In Figs 5 and 6 are shown the results obtained when the colorimeter reading of the aliquot

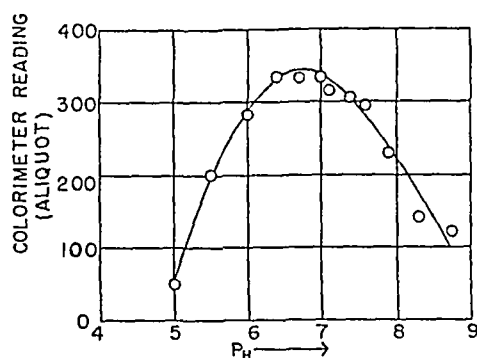


FIG 7

FIG 7 pH optimum of crystallized aldolase at 25°. Klett-Summerson colorimeter, Filter 56

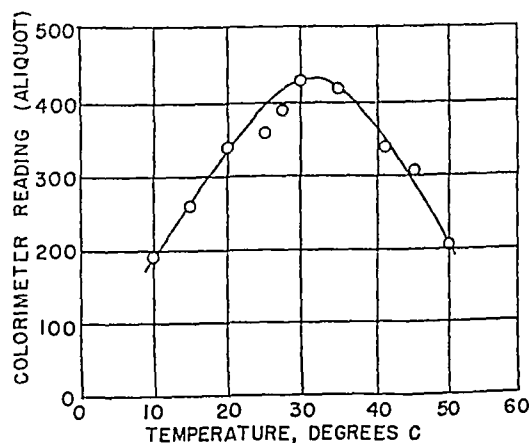


FIG 8

FIG 8 Temperature optimum of crystallized aldolase at pH 7.2. The optimum lies between 30–35° at this pH. Klett-Summerson colorimeter, Filter 56

is plotted against the amount of enzyme employed for crystallized aldolase, isolated liver cell nuclei, and whole liver homogenate. The curve is linear for crystallized aldolase, but it is not linear for nuclei or liver. Hence, in measuring aldolase in nuclei or liver, amounts of material (on a dry weight basis) must not be greater than a specified amount (2 mg) if a linear dilution curve is to be expected. A substrate concentration of 0.07 M was used in these experiments.

*pH Optimum*—All of the above experiments were carried out at a pH value of approximately 7.3. This pH is reasonably close to the optimal pH value for crystallized aldolase, as can be seen from Fig 7, in which the colorimeter reading for the aliquot is plotted against pH. This curve is not in agreement with the curve published by Heiber and collaborators (3).

*Effect of Temperature*—The results of experiments showing the effect of temperature on the activity of aldolase are plotted in Fig 8. It can be

seen that the enzyme has a rather low temperature optimum at pH 7.2 according to our results. The temperature optimum no doubt would depend upon the pH, since the enzyme is more stable in slightly alkaline than in acid solutions.

In order to be sure that our low temperature optimum was not caused by thermal destruction of the reaction products, a point on the activity-temperature curve was checked by heating the enzyme alone for 15 minutes at 40° and then cooling it and running the reaction at 25°. Although this procedure theoretically should not lead to exactly the same results as running the reaction at 40° for 15 minutes with substrate present, the actual results were almost identical. This experiment indicates that the deleterious effect of temperature is on the enzyme and not on the reaction products.

Our temperature optimum for aldolase does not agree with the temperature optimum reported by Herbert *et al.* (3). The latter authors found a much higher optimum.

The  $Q_{10}$  of aldolase as calculated from the ascending branch of the curve is about 1.8. This  $Q_{10}$  value is lower than the value reported by Warburg and Christian (1), from whose data concerning turnover numbers at 20° and 38° we can calculate a  $Q_{10}$  of about 2.8.

#### *Turnover Number for Crystalline Aldolase*

In order to calculate an approximate value for the turnover number of aldolase, it was necessary first to relate the color produced in our method to the moles of fructose diphosphate decomposed into triose phosphate. This was accomplished by allowing 0.1 ml. of strong aldolase to act upon 0.07 M fructose diphosphate for 15 minutes at 25° in order to decompose a sufficiently large portion of the substrate to yield a conveniently measurable amount of phosphate easily hydrolyzable with alkali. An aliquot of the same reaction mixture was then diluted and the color given by the Barker-Summerson reagent (as described above) was measured. Another aliquot was taken for a determination of phosphate easily hydrolyzable by alkali. In this way it was estimated that, if a calculated reading of 2880 were to be obtained from the one-fifth aliquot used for the Barker-Summerson reagent as described above, an amount of easily hydrolyzable phosphate equivalent to 0.2 mg. of phosphorus would have been produced by conversion of the corresponding amount of fructose diphosphate to triose phosphate. Since 4.34 mg. of phosphorus are the total amount present in 1 ml. of 0.07 M fructose diphosphate (the amount used in carrying out the aldolase determination), the percentage decomposition of the substrate equivalent to 0.2 mg. of phosphorus from easily hydrolyzable phosphate or to an

aliquot reading of 2880 from the Barker-Summeison reagent is  $(0.2/4.34) \times 100 = 4.61$  per cent (This represents approximately the value of alkali-labile phosphate produced at equilibrium, since the value was not increased by using more enzyme)

From Fig 5 it can be seen that 1  $\gamma$  of purified aldolase would be equivalent to a colorimeter reading of about 165 from the aliquot used for the Barker-Summeison reagent. This reading of 165 would then correspond to  $(4.61/2880) \times 165 = 0.264$  per cent decomposition of the substrate. This is equal to  $7 \times 10^{-5} \times 2.64 \times 10^{-3} = 18.48 \times 10^{-8}$  mole of fructose diphosphate decomposed, since there is  $7 \times 10^{-5}$  mole of this substance in 1 ml. of a 0.07 M solution.

Therefore in 15 minutes, under the conditions used by us in measuring aldolase,  $18.48 \times 10^{-8}$  mole of fructose diphosphate is decomposed by 1  $\gamma$  of crystallized aldolase, which is equivalent to a decomposition of  $(18.48 \times 10^{-8})/15 = 1.23 \times 10^{-8}$  mole per minute.

If we assume a molecular weight of 150,000 for aldolase, 1  $\gamma$  is equivalent to  $(1 \times 10^{-6})/(1.5 \times 10^5) = 6.66 \times 10^{-12}$  mole of aldolase. Since 1  $\gamma$  or  $6.66 \times 10^{-12}$  mole is equivalent to a colorimeter reading from the aliquot of 165, which is equivalent to a decomposition of  $1.23 \times 10^{-8}$  mole of fructose diphosphate per minute, it can be seen that the number of moles of fructose diphosphate decomposed by 1 mole of aldolase per minute *under the conditions of our determination* (i.e., 25° and 0.07 M substrate concentration) is  $(1.23 \times 10^{-8})/(6.66 \times 10^{-12}) = 0.185 \times 10^4 = 1850$ . This gives a turnover number of 2300 at 30°, which agrees with the value reported by Cori and collaborators (2).

### *Inhibition of Aldolase by Copper*

Heibert *et al.* (3) reported that aldolase is inhibited by copper. We have confirmed this statement, but we do not find that aldolase is especially sensitive to this metal. For 90 per cent inhibition it is necessary to have approximately  $7 \times 10^6$  atoms of divalent copper per molecule of aldolase, assuming a molecular weight of 150,000 for the latter enzyme.

An enzyme which is very sensitive to a heavy metal requires a much lower ratio of metal to enzyme. For instance, Sumner and Myrback (11) found strong inhibition of urease by silver nitrate at a ratio of about 15 atoms of silver to 1 molecule of urease.

The results of our experiments on the inhibition of aldolase by cupric sulfate are shown in Fig 9.

### *Analyses of Tissues for Aldolase*

Our method for aldolase is sufficiently good to be applied to liver, spleen, kidney, pancreas, tumor, plasma of tumor-bearing animals, and isolated

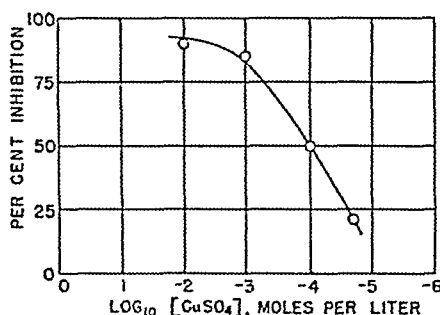


FIG 9 Inhibition of crystallized aldolase by  $\text{CuSO}_4$ . At 90 per cent inhibition, there are  $6.7 \times 10^5$  atoms of copper per molecule of aldolase if a molecular weight of 150,000 is assumed for the latter. Klett-Summerson colorimeter, Filter 56

TABLE I  
*Aldolase Content of Tissues and Isolated Cell Nuclei*

The aldolase activity is expressed as micrograms of aldolase (dry weight) per mg of tissue (dry weight). In the last two columns the results are expressed as micrograms of aldolase (dry weight) per ml of serum. The pairs represent analyses on the same tissue sample, the groups or single values refer to tissues from different animals.

	Muscle		Walker carcinoma 256	Kidney		Liver		Rat spleen	Isolated cell nuclei* of rat liver	Isolated cell nuclei of kidney		Serum	
	Rabbit	Rat		Dog	Rat	Human	Rat			Dog†	Human	Normal rat	Rat bearing Walker carcinoma 256
	25.4	36.0	2.2	1.9	1.7	0.6	0.8	0.5	0.3	0.4	0.2	0.5	6 (Small tumor)
	28.3	33.4	2.3	1.6	1.8	0.7	0.7		0.3				
		31.3	7.4	1.0	1.6		0.8		0.3	0.3			6 (Large tumor)
		29.7	7.4	1.0	1.7		0.8		0.3				
		27.5	3.6						0.4				
		27.5	3.3						0.3				
Average	26.9	30.9	4.4	1.4	1.7	0.7	0.8		0.3	0.4			6

\* The activity per dry weight of aldolase in isolated rat liver nuclei is roughly 40 per cent of its activity in whole rat liver.

† The activity per dry weight of aldolase in isolated dog kidney nuclei is roughly 20 per cent of its activity in whole dog kidney.

‡ This dog had a badly infected fore leg and the kidneys showed some signs of pathology macroscopically.

cell nuclei from liver, kidney, and pancreas. The activities of aldolase present in these tissues per unit of dry weight as measured in terms of

crystalline aldolase prepared by us<sup>6</sup> (which is not necessarily pure) are shown in Table I. We have found that nuclei isolated from normal rat liver contain aldolase in about 40 per cent of the activity, per unit of dry weight, of the corresponding value for aldolase in whole liver homogenate. The activity per unit of dry weight of aldolase in nuclei isolated from dog kidney by a modification of our original method for rat liver cell nuclei (11) is about the same as that of rat liver cell nuclei, whereas the activity per unit of dry weight of aldolase in nuclei isolated from sheep pancreas appears to be negligible. (Work describing the preparation of nuclei from kidney and pancreas will appear elsewhere.) Aldolase decays rapidly in isolated cell nuclei kept at room temperature, but there is negligible decay over a period of 24 hours if the nuclei are kept at a temperature of about 3°.

Walker carcinoma 256 contains aldolase in about 3 times as high an activity per unit of dry weight as the corresponding value for aldolase in normal rat liver. The activity per unit of dry weight of aldolase in the plasma of rats bearing large Walker carcinomas can be considerably higher than that for aldolase in normal rat plasma. This is in agreement with the work of Warburg and Christian (12). However, the activity per unit of dry weight of aldolase in muscle far exceeds the activity of aldolase in any other type of tissue studied by us, as might be expected from the work of Taylor, Green, and Cori (2).

#### DISCUSSION

All of the above results show that aldolase can be measured in isolated liver cell nuclei and whole liver homogenate by our new method, as well as in solutions of the crystallized enzyme. If nuclei or tissue are employed, the time of incubation with substrate must be limited to 15 minutes, and for linear dilution curves not more than 2 mg of tissue (on a dry weight basis) can be used in a determination. The new method thus apparently can be applied rather generally, if the limitations mentioned above are kept in mind.

It may be of interest to compare our method for aldolase determination with the methods employed by previous investigators (Herbert *et al* (3), Warburg and Christian (1)). In the method of Herbert *et al*, the rate of formation of hydrolyzable phosphate is used in determining the enzyme. The hydrolyzable phosphate is attributable to the triose phosphate that is formed. The phosphate is hydrolyzed from the triose phosphate by exposure to N "soda" for 20 minutes at room temperature. Cyanide is added to avoid reversal of the reaction by fixing the triose phosphate as cyanohydrin.

<sup>6</sup> We wish to express our sincere appreciation to Dr. G. T. Cori for helpful advice concerning the preparation of crystalline aldolase.

In this method, anything which might liberate inorganic phosphate from the substrate (hexose diphosphate), such as phosphatase, can be expected to interfere with the determination. The pH optimum reported by these investigators for aldolase is at approximately 9.0, but their curve has a slight shoulder in the neighborhood of pH 7.5, which is not far from the optimal pH for aldolase as determined by our method. It seems possible that their high pH optimum may have been obtained because of interfering phosphatases, or because of non-enzymatic hydrolysis of triose phosphate at high pH values, or possibly because of some effect of the HCN which was present. Although the method of Herbert *et al.* may be satisfactory for purified aldolase, it seems doubtful whether it could be applied very successfully to a determination of aldolase in tissues other than possibly muscle, which contains a very high concentration of aldolase.

The method of Warburg and Christian (1) for determining aldolase is more complicated. These investigators add crystallized 3-phosphoglyceraldehyde dehydrogenase and coenzyme I and then determine spectrophotometrically the rate of reduction of the coenzyme I. The experiment is arranged so that the aldolase concentration is the limiting factor. This method is so complicated that its use in semiroutine determinations of aldolase would be difficult. Moreover, although the method undoubtedly is very satisfactory for pure aldolase, it may be less accurate when applied to whole tissue because of competing enzymes. For instance, an enzyme is present in many tissues which destroys coenzyme I and which therefore might interfere.

It will be noticed that our method for determining aldolase is more direct than the method of Warburg and Christian, since triose phosphate itself is measured. Our method also is very sensitive, since the immediate product of the reaction is measured by a very sensitive test. This makes it possible to use a relatively high substrate concentration in order to minimize effects of possible loss of substrate from competing reactions. The effects of enzymatic reactions which could remove or destroy the reaction products also are minimized, since the small concentrations of reaction products necessary to be measured are attained very quickly. Interfering enzyme reactions involving the reaction products (triose phosphates) should be slow compared to the reaction catalyzed by aldolase when the concentration of substrate (hexose diphosphate) is high compared to the concentration of products. High substrate concentration also simplifies the kinetics of the reaction by setting up a zero order reaction. The chief disadvantage of our method is that relatively high blank values often are obtained.

In our method so little substrate is hydrolyzed that we do not find it necessary to add cyanide to avoid interference by reversal of the reaction.

It was found that at least 4.61 per cent of the fructose diphosphate must be split at equilibrium, with a substrate concentration of 0.07 M at a temperature of 25° (See the work reported above under "Turnover number for crystalline aldolase") However, so little substrate is hydrolyzed in our method of determining aldolase (less than 1.0 per cent in nearly all cases) that reversal of the reaction is of no consequence even though at our higher substrate concentration less substrate is split at equilibrium than in the method of Herbert and collaborators (3)

As applied to tissues, our method for determining aldolase apparently gives lower results in terms of micrograms of enzyme per mg of tissue than the method of Warburg and Christian (1), but the ratios of the concentration of aldolase in muscle to its concentration in other tissues are similar for both methods

#### SUMMARY

1 A new method for determining aldolase has been devised which depends upon a colorimetric determination of the triose phosphate produced by the action of this enzyme

2 In all probability, both dihydroxyacetone phosphate and 3-phosphoglyceraldehyde contribute strongly to the production of color in this procedure, so that interference by isomerase is probably negligible

3 Practical studies of the kinetics of aldolase have been carried out by means of the new method The pH optimum has been found to lie in the neighborhood of 7.0 instead of 9.0 as reported by Herbert *et al* (3)

4 The new method has been applied to a determination of aldolase in liver, kidney, spleen, pancreas, muscle, tumor, and plasma of tumor-bearing animals, as well as to cell nuclei isolated from rat liver, dog kidney and sheep pancreas Aldolase has been found in very appreciable concentration in cell nuclei isolated from liver and kidney, but it was not detected in nuclei isolated from pancreas The aldolase of the plasma of the tumor-bearing rats was considerably higher than normal, confirming results obtained by Warburg and Christian (12) using other tumors

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# THE METABOLIC FATE OF ESTRONE IN BILE FISTULA DOGS\*

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It has been repeatedly demonstrated that, following the administration of estrogen to human or animal subjects, the hormone disappears rapidly from the organism and only a small quantity of the original compound or its metabolites can be recovered in crystalline form from the urine (2). On the other hand, Cantarow *et al* (3) detected in the bile of dogs with external bile fistula as much as 90 to 95 per cent of the biological activity of the estrone or  $\alpha$ -estradiol which had been injected intravenously. Since bioassay had been performed directly on the bile without resort to fractionation procedures, it was thought desirable to pursue the problem of biliary excretion of estrogens along chemical lines. Accordingly, a total quantity of 1.476 gm of estrone acetate (containing 1.278 gm as estrone equivalent to 18,000,000 mouse units of estrogenic activity) was dissolved in oil and injected intramuscularly into three external bile fistula dogs. The bile, urine, and feces were collected and extracted, 79 mg of estrone and 18 mg of  $\alpha$ -estradiol were isolated in crystalline form from the bile, considerably less estrogenic material as determined by bioassay was contained in the urine or feces. A detailed report is given below.

Table I lists the estrogenic activity of the phenolic material derived from the bile, urine, and feces respectively, bioassay of unfractionated bile was also performed. Inspection shows that (a) considerably more estrogen was excreted in the bile than in the urine, a small but significant amount of estrogen appeared in the feces, (b) most of the estrogenic material of the bile and of the feces was present in a free or uncombined form, much of the estrogenic material in the urine was present in conjugated form, but the ratio of free to conjugated estrogen varied considerably.

Table II indicates the distribution of the estrogenic activity of the various extracts between the ketonic and non-ketonic weakly acidic phenols and the strongly acidic phenols. Hydrolysates of the conjugated phenolic material of the bile and feces were not partitioned because of the

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low content of estrogen It may be seen from Table II that (a) almost all of the estrogenic activity of the extracts (bile, urine, and feces) resided in the weakly acidic phenols, and (b) a variable proportion (22 to 88 per cent)

TABLE I

*Recovery of Estrogenic Activity after Intramuscular Injection of Estrone Acetate into External Bile Fistula Dogs*

Dog No	Estrone content equivalent of hor- mone injected*	Source extracted	Post injection collec- tion period	Estrogenic activity†		
				Untreated bile	Free phenols	Conjuga- ted phe- nols (after hy- drolysis)
	mg		days	mouse units	mouse units	mouse units
69 (male), poor liver function‡	346 (4,800,000)	Bile	1 + 2	492,000	180,000	1,000
			3 + 4	165,000	67,000	<160
		Urine	1 + 2		54,000	23,000
			3 + 4		3,000	4,000
		Feces	1 + 2 + 3 + 4		13,000	200
B72 (female), good liver function‡	499 (7,000,000)	Bile	1 + 2	1,900,000	1,400,000	17,000
			3 + 4	400,000	480,000	500
			5	150,000	160,000	
		Urine	1 + 2§		10,000	20,000
			3 + 4		150,000	20,000
B75 (female), good liver function‡	433 (6,100,000)	Feces	2 + 3 + 4		14,000	550
		Bile	1	830,000	922,000	
			2	80,000		
		Urine	1		16,000	28,000
2			5,000	10,000		
Feces	1			9,000	120	
		2		700	100	

\* Estrogenic activity (in parentheses, mouse units) based on estrone content (oil injection)

† Bioassay was performed on spayed adult mice by use of a vaginal smear technique based on that described by Allen and Doisy (24), aqueous dilutions of bile were injected, whereas the phenol fractions (bile, urine, or feces) were first dissolved in oil. Factors which may account for discrepancies in the bioassay value of untreated bile and that of the phenolic material contained therein have been discussed in a previous report (12). Estrogenic activity could not be detected in the feces of normal dogs.

‡ Indicated by studying the excretion of bromosulfathalein in the bile.

§ Contamination with feces due to diarrhea during the 1st day of collection.

of the total biological activity was due to the non-ketonic fraction of the weakly acidic phenols.

Table III gives the percentage recovery in the bile of the biological activity (mouse units) of the hormone injected, as well as that of estrogenic material (mg of estrone plus  $\alpha$ -estradiol).

TABLE II

*Distribution of Estrogenic Activity\**

The results are expressed in per cent for each collection period

Dog No	Source extracted	Post injection collection period	Weakly acidic phenols		Strongly acidic phenols†	
			Ketonic	Non ketonic		
69	Bile (free phenols)	days 1 + 2	78	22	<1	
		3 + 4	42	58		
	Urine " "	1 + 2	34	62	5	
		3 + 4	33	56	11	
	" (conjugated phenols after hydrolysis)	1 + 2	21	74	5	
		3 + 4	16	80	4	
	Feces (free phenols)	1 + 2 + 3 + 4	10	88	2	
	B72	Bile " "	1 + 2	71	29	<1
3 + 4			37	62	1	
5			11	88	<1	
Urine " "		1 + 2	73	24	<3	
		3 + 4	25	73	2	
		Feces " "	2 + 3 + 4	7	88	5
B75		Bile " "	1	26	74	0 2

\* Determined by bioassay (see foot-note, Table I)

† The actual values may be even lower, since after further partitioning of pooled strongly acidic phenols (8200 mouse units) of the bile, the 0.3 M Na<sub>2</sub>CO<sub>3</sub> soluble phenols assayed only 1200 mouse units. Similarly, the corresponding fractions of the urine after pooling assayed 7500 mouse units, but after further partitioning assayed only 3200 mouse units.

TABLE III

*Percentage Recovery in Bile of Estrogenic Activity and Estrogenic Substance (for Entire Collection Period)*

Dog No	Based on bioassay of		Based on calculated* content of estrone and $\alpha$ estradiol
	Bile directly (aqueous injections)	Total phenolic material of bile (oil injections)	
69	14	5	4
B72	35	29	22
B75	15	15-16	9-10

\* It is assumed that the biological activity of the bile is due entirely to the above substances. These figures may be calculated from those given in Tables I and II and from the bioassay values for 1  $\gamma$  each of estrone and  $\alpha$ -estradiol in oil which are 14 and 33 mouse units respectively in this laboratory.

It is evident from Tables I and II that most of the biological activity of the bile was due to the weakly acidic phenols (*unhydrolyzed*). This ma-

terial appeared to be suitable for isolation work. Accordingly, the ketonic fractions which were derived from the bile were pooled, 123 mg of crystalline material (1,500,000 mouse units equivalent to 107 mg of estrone) were obtained. The material was recrystallized from alcohol, yielding 79 mg of impure estrone, m p 252–256°, which, on subsequent purification, proved to be identical with an authentic specimen. From the non-ketonic weakly acidic phenols (*unhydrolyzed* bile) there were obtained 118 mg of a semi-crystalline product (1,600,000 mouse units equivalent to 48 mg of  $\alpha$ -estradiol). It yielded 29 mg of digitonin-precipitable material which, on crystallization from alcohol, gave 18 mg of  $\alpha$ -estradiol. The identity of this product was established by ascertaining its specific optical rotation and by a determination of the melting point on admixture with an authentic specimen of  $\alpha$ -estradiol, a dibenzoyl derivative was also prepared which gave the expected carbon and hydrogen values on analysis. The non-digitonin-precipitable material was submitted to chromatographic analysis, but  $\beta$ -estradiol could not be isolated.

#### EXPERIMENTAL

*Collection and Extraction of Bile, Urine, and Feces*—Bile was collected in 24 hour periods, and after adjusting the pH to 6.5 to 7.5, it was immediately extracted by gentle shaking with *n*-butanol, for each 100 ml of bile, 50 ml of solvent were used in the initial extraction, followed by four extractions each with 10 ml portions. The butanol extracts were combined, washed twice with 10 ml portions of water, and evaporated *in vacuo*. The butanol residue was dissolved in 25 ml of water, made distinctly acid to litmus with concentrated HCl, and extracted once with 100 ml and then twice with 50 ml of ether. The ether extracts were combined, back-washed with 10 ml of water, washed successively with dilute sodium bicarbonate and water, and evaporated. The ether residue was dissolved in benzene and extracted four times with equal volumes of *N* NaOH, the alkaline extracts were back-washed with 0.2 volume of benzene. The benzene solution was washed with water and evaporated to give the *neutral fraction (unhydrolyzed bile)*. The alkaline extracts were made acid to Congo red with concentrated HCl and thoroughly extracted with ether in order to obtain the *phenolic fraction (unhydrolyzed bile)*. The *acid fraction (unhydrolyzed bile)* was obtained from the residual bile (after butanol extraction) by acidification to Congo red with concentrated HCl followed by extraction with ether. Some acidic material was also obtained from the sodium bicarbonate washes of the ether-soluble, water-insoluble fraction of the butanol residue described above. The water-soluble, ether-insoluble fraction of the butanol residue was heated on the water bath to remove dissolved ether, after the addition of 10 per cent by volume of concentrated

HCl, the solution was refluxed for 10 minutes, rapidly cooled, and extracted with ether. The *phenolic fraction (hydrolyzed bile)* was obtained from the ether extract in the usual way.

Urine was collected in 24 hour periods and immediately extracted with butanol, the phenolic fractions were obtained as above. Feces were ground with sand and extracted with liberal amounts of alcohol at room temperature. The residue obtained on evaporation of the alcohol extracts was partitioned into "free" phenols and conjugated phenols, the latter were subsequently hydrolyzed.

*Fractionation of Phenolic Material*—The strongly acidic and weakly acidic phenolic fractions were obtained by the method of Mather (4) by distributing the phenols between benzene and 0.3 M  $\text{Na}_2\text{CO}_3$ . Repeated partitioning was necessary in order to achieve effective separations, especially with bile extracts. Troublesome emulsions were often encountered which were broken by long centrifugation. The weakly acidic phenols were separated into ketonic and non-ketonic moieties with the aid of Girard's Reagent T (5).

*Isolation and Identification of Estrone (Unhydrolyzed Bile)*—The ketonic, weakly acidic phenols (*unhydrolyzed bile*) excreted by Dog 69 (1 + 2 + 3 + 4 days), Dog B72 (1 + 2 + 3 + 4 + 5 days), and by Dog B75 (1 day) were pooled. Since the estrogenic content of bile collected subsequently was comparatively small, this material was not included. The pooled material was crystalline, weighed 123 mg, and assayed 1,500,000 mouse units (equivalent by our method of bioassay to 107 mg of estrone). It was recrystallized from alcohol to give 79 mg of impure estrone, m p 252–256°, an additional crop of crystals, m p 225–232°, was obtained from the mother liquors. Repeated crystallization of the former product yielded 45 mg, m p 258–260°,  $[\alpha]_D^{28} = +170^\circ$  (0.752 per cent in dioxane), admixture with authentic estrone, m p 259–260°,  $[\alpha]_D^{29} = +163^\circ$  (in dioxane), gave no depression in melting point. A benzoyl derivative was prepared which melted at 219.5–221° and gave no depression in melting point on admixture with authentic estrone benzoate, m p 222–223°.

*Isolation and Identification of  $\alpha$ -Estradiol (Unhydrolyzed Bile)*—The non-ketonic, weakly acidic phenols corresponding to the ketonic phenols above were pooled. There were obtained 118 mg of a semicrystalline product which assayed 1,600,000 mouse units (equivalent by our method of bioassay to 48 mg of  $\alpha$ -estradiol). After treatment with 400 mg of digitonin by a method described by Huffman *et al* (6), there were obtained 29 mg of digitonin-precipitable material. It crystallized readily from aqueous alcohol to give 18 mg, m p 173–175°,  $[\alpha]_D^{31} = +77^\circ$  (0.710 per cent

<sup>1</sup> All melting points reported here are corrected

in absolute ethanol) Further recrystallization from the same solvent raised the melting point to  $175^{\circ}$ , no melting point depression was observed on admixture of this product with authentic  $\alpha$ -estradiol, m p  $176^{\circ}$ ,  $[\alpha]_D^{30} = +78^{\circ}$  (in absolute ethanol)

A dibenzoyl derivative was prepared from 14 mg of the above product, m p  $173-175^{\circ}$  It was repeatedly crystallized from chloroform-ethanol to give 9 mg, m p  $169.5-170^{\circ}$ , admixture with authentic  $\alpha$ -estradiol dibenzoate, m p  $170-170.5^{\circ}$ , gave no depression in melting point

$C_{32}H_{42}O_4$  Calculated, C 79.96, H 6.72, found, C 79.80, H 6.74

Chromatographic analysis of the non-digitonin-precipitable fraction (58 mg) failed to yield any crystalline products

#### DISCUSSION

Whereas previous evidence (2) for the *in vivo* conversion of estrone into  $\alpha$ -estradiol has been entirely circumstantial, the evidence presented here is based on the isolation of the metabolite in crystalline form It cannot be assumed, however, that the reduction of estrone proceeds in this direction in all mammalian species, especially since  $\beta$ -estradiol but no  $\alpha$ -estradiol was isolated from the urine of rabbits injected with estrone (7), the  $\beta$  isomer was likewise isolated in similar experiments (8, 9) in which  $\alpha$ -estradiol was injected In the present study,  $\beta$ -estradiol could not be isolated from the bile

The reverse process, *viz* the biological conversion of  $\alpha$ -estradiol into estrone, has been clearly demonstrated (2), however, in the case of the dog, the evidence (10, 11) is of an indirect nature It might be inferred that these processes take place in the liver, since estrogens of endogenous<sup>2</sup> and exogenous origin have been isolated from bile, some support for this hypothesis has been obtained from *in vitro* experiments (13, 14)

It appears questionable from our data that the dog is capable of transforming estrone into estriol to any significant extent<sup>3</sup> On the other hand, Longwell and McKee (15) detected a significant degree of estrogenic activity in the 0.3 M  $Na_2CO_3$ -soluble, benzene-insoluble phenolic fraction of bile obtained from dogs injected with small quantities of estrone (Of the native estrogens, estriol alone is partitioned in this manner (4, 16)) Pearlman *et al* (11) likewise detected some biological activity in similar material obtained from dogs injected with  $\alpha$ -estradiol

<sup>2</sup> Estrone has recently (12) been isolated from the bile of pregnant cows, it is the major estrogen of the bile

<sup>3</sup> See foot note, Table II It is quite likely that most of the biological activity exhibited by the strongly acid phenols was, in our experiments, due to slight contamination with the highly active, weakly acidic phenols

Our experience, in common with that of most investigators working in the field of estrogen metabolism, indicates that a major portion of the estrogenic substance administered cannot be accounted for in the excreta as biologically active material. Chromatographic analysis of the neutral material<sup>4</sup> and of the free acids<sup>5</sup> of the pooled specimens of unhydrolyzed bile was undertaken, but none of the hypothetical products (2) of estrogen inactivation was isolated.

Our data (see Table III) on the percentage recovery in the bile of the biological activity of the injected hormone are in closer agreement with the data of Longwell and McKee (15) than with those of Cantarow *et al* (3). The former authors observed a biliary excretion ranging from 13 to 80 per cent, whereas the latter authors reported an almost quantitative recovery of hormonal activity. Pearlman *et al* (11) reported that 10 per cent or possibly 26 per cent (bioassays performed independently in the two laboratories) of the biological activity of the injected  $\alpha$ -estradiol was detected in the bile. In a study which apparently was the first of its kind on this subject, Stamler (18) reported a biliary excretion of "not less than 13 per cent" of the estrone injected. It is difficult to explain these discrepancies. Cantarow *et al* (3) have emphasized the necessity of insuring a satisfactory state of nutrition and liver function in bile fistula dogs, since otherwise relatively insignificant hepatic functional defects may have a profound influence upon the metabolism of the steroid hormones.<sup>6</sup>

Considerably more estrogen is excreted in the bile than in the urine following the administration of estrone acetate (see Table I). On the other hand, Longwell and McKee (15) reported a biliary excretion ranging between 13 to 80 per cent of the injected hormone and a urinary excretion between 6.4 and 13.5 per cent. Perhaps no great significance can be attached to this point of difference if one considers the unphysiological dose of estrogen employed in our experiments which, in this instance, were designed primarily to ascertain the nature of the metabolites of estrone rather than to obtain information concerning the physiological transport of estrogen. Yet, in a study by Cantarow *et al* (21) in which a comparatively small amount of estrone (250,000 I.U.) was injected intravenously, it

<sup>4</sup> The neutral material of unhydrolyzed bile contained less than 20,000 mouse units. This precludes the possibility that estrone acetate was present and attests to the quantitative removal of unesterified estrogen from this fraction.

<sup>5</sup> The free acids of unhydrolyzed bile contained only 3300 mouse units. This makes it appear unlikely that any but trace amounts of the highly active compounds of the doisyonic acid series were present. It had been suggested that such compounds might occur naturally (17).

<sup>6</sup> The subject of nutrition and endocrinology has recently been reviewed by Hertz (19) and also by Biskind (20).



was reported that the amount of estrogen in the urine was much less than that in the bile

The fact that a small but none the less significant degree of estrogenic activity is exhibited by the phenolic material of the feces of *bile fistula* dogs (all the bile draining externally) is an indication that estrogen entered the bowel from the blood stream by passage through the intestinal wall. Perhaps this mechanism operates under normal physiological conditions, it would account, in part at least, for the estrogen content of the feces of pregnant women (22) or of pregnant cows (23)

Our findings with regard to the chemical nature of the estrogens of dog feces parallel (perhaps fortuitously) those obtained by Levin (23) in his investigation of the fecal estrogens of pregnant cows in that (a) the estrogen is present chiefly in a free or unconjugated form, and (b) the biological activity resides chiefly in the non-ketonic, weakly acidic phenols

#### SUMMARY

Massive doses of estrone, as the acetate, were injected intramuscularly into three external bile fistula dogs. A small quantity of estrone and of  $\alpha$ -estradiol was isolated from the pooled bile specimens. By comparison, the urine and feces contained much less estrogen as determined by bioassay. Most of the estrogenic substance administered cannot be accounted for in the excreta.

The implications of these findings are discussed.

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# PARTIAL SYNTHESIS OF ETIOCHOLENE-9-OL-3( $\alpha$ )-ONE-17

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The isolation of a new steroid from human urine has recently been reported (1). A study of the properties of this substance led to the surmise that it possessed the structure I, etiocholene-9-ol-3( $\alpha$ )-one-17. In order to aid in the confirmation of this hypothesis, the partial synthesis of I was undertaken.

Vigorous catalytic reduction of etiocholanol-3( $\alpha$ )-dione-11,17 acetate (II) gave etiocholanetriol-3( $\alpha$ ),11( $\beta$ ),17( $\alpha$ ) acetate-3 (III). Aluminum tertiary butoxide in acetone effected the partial oxidation (2) to etiocholanediol-3( $\alpha$ ),11( $\beta$ )-one-17 acetate-3 (IV). Phosphorus oxychloride in pyridine smoothly dehydrated (3) III to the acetate of the desired  $\Delta^9$  derivative, from which I was obtained by saponification.

## EXPERIMENTAL

All melting points are corrected. Rotations were taken in acetone,  $c \sim 1.0$ .

*Etiocholanetriol-3( $\alpha$ ),11( $\beta$ ),17( $\alpha$ ) Acetate-3 (III)*—A solution of 2.0 gm of etiocholanol-3( $\alpha$ )-dione-11,17 acetate in 50 cc of acetic acid was shaken under hydrogen with 800 mg of previously reduced platinum (Adams' catalyst). After 3 hours the reduction was complete. The solution was then filtered, concentrated to dryness *in vacuo*, dissolved in ether, washed with dilute sodium carbonate and with water, and concentrated to a small volume on the steam bath. The addition of petroleum ether gave 1.85 gm of crystals, m p 183–185°. After several recrystallizations from dilute alcohol and from acetone-petroleum ether, the melting point was 198–200°.

*Analysis*— $C_{27}H_{44}O_4$ . Calculated, C 71.96, H 9.78, found, C 72.21, H 9.73.

*Etiocholanetriol-3( $\alpha$ ),11( $\beta$ ),17( $\alpha$ )*—Saponification of a sample of the monoacetate (III) gave the free triol, which, upon recrystallization from acetone and from benzene, melted at 205–206°.

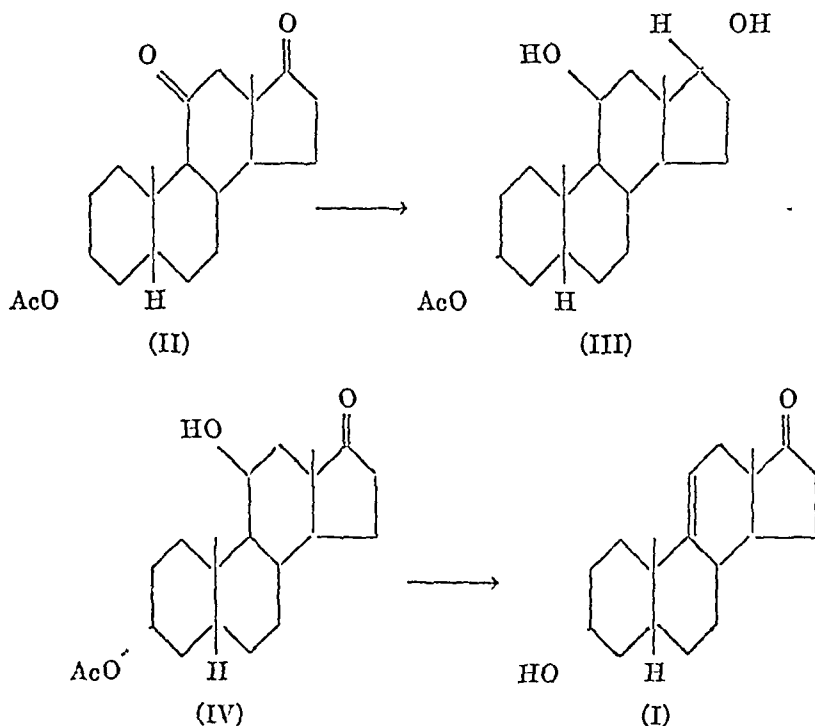
*Analysis*— $C_{27}H_{46}O_3$ . Calculated, C 73.97, H 10.46, found, C 73.91, H 10.38.

*Etiocholanetriol-3( $\alpha$ ),11( $\beta$ ),17( $\alpha$ ) Diacetate-3,17*—A solution of 35 mg of the monoacetate (III) in 0.5 cc of pyridine and 0.5 cc of acetic anhydride was heated on the steam bath for 15 minutes. The addition of water gave

the diacetate, which melted, after recrystallization from ether-petroleum ether, at 161–162°

*Analysis*— $C_{27}H_{46}O_5$  Calculated, C 70.37, H 9.25, found, C 70.48, H 9.22

*Etiocolanediol-3( $\alpha$ ),11( $\beta$ )-one-17 Acetate-3 (IV)*—A mixture of 1.5 gm of the monoacetate (III), 80 cc of dry benzene, 40 cc of anhydrous acetone, and 3.0 gm of aluminum isopropoxide was refluxed overnight. Most of the solvent was then removed *in vacuo*, the residue taken up in ether, washed with dilute hydrochloric acid and with water, and the ethereal solution concentrated to dryness. The residue was dissolved in 15 cc of



methanol and treated with 2.0 gm of Girard's reagent together with 0.8 cc of acetic acid. After refluxing for 30 minutes, the solution was concentrated to half volume *in vacuo*, then poured into a mixture of ice water and ether in a separatory funnel. After thorough mixing the aqueous layer was removed and acidified with 20 cc of 4 N hydrochloric acid.

After standing several hours at room temperature the acidified aqueous layer was extracted with two 200 cc portions of ether. The ethereal layer was washed with dilute sodium carbonate, then with water, and concentrated to dryness. The residue (500 mg) was then heated with pyridine-acetic anhydride for 10 minutes on the steam bath to replace acetoxy groups lost by hydrolysis. (This usually amounts to 10 per cent when the Girard complex is worked up in the manner described above.) Careful

addition of water then gave 440 mg of crystals, m p 223–229° This material was chromatographed over 8 gm of alumina (acid-washed) The crystalline fractions eluted with ether and ether-chloroform mixtures were combined and recrystallized from acetone and from benzene, which gave 222 mg of etiocholenediol-3( $\alpha$ ),11( $\beta$ )-one-17 acetate-3, m p 237–238° For analysis a sample was dried *in vacuo* at 140°

*Analysis*—C<sub>19</sub>H<sub>26</sub>O<sub>4</sub> Calculated, C 72.40, H 9.26, found, C 72.50, H 10.04

*Etiocholenediol-3( $\alpha$ ),11( $\beta$ )-one-17*—A sample of the acetate (IV) was saponified and gave the free diolone Recrystallized from acetone, it melted at 237.5–239° A mixture with the acetate melted at 205–220°

*Analysis*—C<sub>19</sub>H<sub>26</sub>O<sub>3</sub> Calculated, C 74.41, H 9.87, found, C 74.44, H 9.90

*Etiocholene-9-ol-3( $\alpha$ )-one-17 (I)*—A solution of 170 mg of etiocholenediol-3( $\alpha$ ),11( $\beta$ )-one-17 acetate in 1 cc of dry pyridine was treated with 0.2 cc of phosphorus oxychloride After standing at room temperature overnight, the solution was diluted with water and extracted with ether The washed ethereal solution was concentrated to dryness and the residue (160 mg) chromatographed The fraction more easily eluted consisted of a colorless oil (146 mg) which could not be obtained crystalline In addition 10 mg of starting material were obtained The non-crystalline fraction was dissolved in 5 cc of methanol and treated with a solution of 200 mg of potassium carbonate in 2 cc of water After standing at room temperature overnight, the solution was concentrated to half volume *in vacuo* and water added The crystalline precipitate weighed 114 mg and melted at 169–171° After recrystallization from dilute alcohol and from ether-petroleum ether, it melted at 171°,  $[\alpha]_D^{25} = +155.5^\circ \pm 2^\circ$

*Analysis*—C<sub>19</sub>H<sub>26</sub>O<sub>2</sub> Calculated C 79.13, H 9.85  
Found " 79.61, 79.59, H 9.87, 10.15

Grateful acknowledgment is made to Miss Jean Andrews for technical assistance For stimulating suggestions concerning this work the author is indebted to Dr K Folkers and Dr R T Major of these laboratories and to Dr E S Wallis of Princeton University The microanalyses reported herein were carried out by Messrs R Boos, E Thornton, J McGregor, and R Funk

#### SUMMARY

Etiocholene-9-ol-3( $\alpha$ )-one-11 has been prepared by partial synthesis from etiocholanol-3( $\alpha$ )-dione-11,17

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# STUDIES IN PROTEIN METABOLISM WITH COMPOUNDS LABELED WITH RADIOACTIVE CARBON

## I METABOLISM OF DL-TYROSINE IN THE NORMAL AND TUMOR BEARING RAT\*

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In 1939 Schoenheimer, Ratner, and Rittenberg (1) employed DL-tyrosine labeled with heavy nitrogen ( $N^{15}$ ) to study the metabolism of this amino acid and the fate of its amino nitrogen in rats. The present investigation deals with the metabolism in rats of radioactive DL-tyrosine (tyrosine\*), labeled in the  $\beta$  position with the long lived  $C^{14}$  isotope of carbon (2) <sup>1</sup>

The labeling of the carbon skeleton made it possible to measure the incorporation of tyrosine into tissue proteins and the conversion of this amino acid into other compounds. The moderately high radioactivity of the tyrosine\* permitted the administration of single small doses (5 mg), well within the normal physiological range, and the subsequent measurement (with an accuracy of about 5 per cent) of fractions of the order of 0.001 to 0.0001 part of such doses.

The present experiments revealed that the carbon chain of the tyrosine, unlike the amino nitrogen, does not contribute significantly to the formation of other amino acids.

### EXPERIMENTAL

*Measurement of Radioactivity*—Because of the very soft  $\beta$ -rays emitted in the disintegration of  $C^{14}$ , it was necessary to use a supported thin mica window bell type tube (3) with the Geiger-Muller counter for the measurements. The samples (generally 3 to 30 mg) were collected on small filter papers with the aid of suction flasks of the type described by Melchior and Tarver (4). In certain cases, e.g. in the preparation of tyrosine\* standards, solutions or suspensions were evaporated to dryness on thin aluminum disks. In order to compensate for errors due to self-absorption of radiation, the standards were mixed with amounts of ordinary tyrosine or protein comparable to the weights of the unknown samples measured.

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† National Cancer Institute Senior Research Fellow.

<sup>1</sup> The authors thank Dr. M. Calvin and Dr. J. C. Reid of the Radiation Laboratory of the University of California for generously furnishing this isotopic tyrosine.



*Tyrosine\* Solutions*—5 mg samples of tyrosine\* were dissolved in 1 ml portions of 0.68 per cent sodium carbonate solution. A small accurately measured fraction of each solution was used to prepare the standards already mentioned. The radioactivity, as measured in the Geiger counter, amounted to approximately 70,000 counts per minute per mg. of tyrosine\*.

*Experimental Animals*—The experiments were performed with five male rats weighing about 200 gm. Two of the animals had bilateral lymphosarcomas. These tumors were month-old transplants from a spontaneous sarcoma.<sup>2</sup> After a 12 hour fast, 1 ml. of the tyrosine\* solution was injected into the jugular vein of each animal. During subsequent periods of specified duration, the urine was collected, and the animals were fed the usual diet. Finally the rats were sacrificed and the body tissues analyzed as described below.

*Preparation of Proteins*—The blood, obtained by cardiac puncture, was collected and centrifuged. The various organs and tissues were excised, and the remaining "carcass" was ground and sampled.

The tissue fractions were ground in a Potter type (glass) homogenizer in the presence of excess 10 per cent trichloroacetic acid. The resulting precipitates were collected by centrifugation, washed with 5 per cent trichloroacetic acid, suspended twice in warm acetone and lastly in ether, and dried in air.

*Isolation of Tyrosine*—The protein samples were autoclaved with 15 parts of 6 N hydrochloric acid in sealed tubes for 20 hours at 15 pounds pressure. Then the hydrolysates were freed of hydrochloric acid by evaporation to dryness *in vacuo*, followed by treatment with silver carbonate. Decolorizing carbon was also employed in the course of this procedure. The solutions were concentrated to small volumes, adjusted to pH 5.7 with alkali, and the tyrosine crystallized out at 5°.

The crude tyrosine was collected by centrifugation and recrystallized three times from boiling water. The purified products were transferred to filter papers with the aid of alcohol washings and dried. The radioactivity of each sample was then determined, and finally the tyrosine was redissolved in acid and determined colorimetrically by the Bernhart modification of the Millon-Weiss reaction (5). The colorimetric values were about 5 to 10 per cent lower than the corresponding weights of the tyrosine preparations.

*Dicarboxylic Amino Acids*—After removal of the crude tyrosine from the hydrolysates, saturated hot barium hydroxide solution was added to the latter until pH 9.5 to 10.0 was reached. After centrifuging to remove small amounts of barium carbonate, the solutions were treated with 3 volumes of alcohol. The resulting precipitates of the crude barium salts of glutamic

\* These rats were kindly supplied by Dr. H. B. Jones of the Radiation Laboratory.

and aspartic acids were collected by centrifugation and then redissolved in water. In order to remove completely any traces of radioactive tyrosine, a moderate amount of the L-tyrosine carrier (about 50 mg per gm of original protein) was dissolved in each solution. Then 6 N sulfuric acid was added to pH 5.7 to remove the barium and to permit subsequent crystallization of the tyrosine carrier. This crystallization of the tyrosine was again accomplished by concentrating the solutions and cooling them to 5°.

The dicarboxylic amino acids were reprecipitated as before with barium hydroxide, transferred to filter papers, washed with alcohol, dried, and counted for radioactivity. Inasmuch as the  $C^{14}$  content of the samples was always quite low, the isolation of pure glutamic and aspartic acids was not undertaken.

*Arginine*—After removal of the crude dicarboxylic acid fractions, the alcoholic solutions were concentrated to small volumes to remove the alcohol. Then 6 N sulfuric acid was added to pH 4, and the resulting barium sulfate precipitates removed by centrifugation. To the solutions was added flavianic acid, whereupon arginine diflavanate crystallized out. The crystals were collected on filter papers, washed with water, dried, and tested for activity.

*Residual Amino Acids*—The filtrates from the arginine diflavanates were freed of flavianic acid by acidification with hydrochloric acid and extraction with *n*-butanol. The colorless aqueous solutions were evaporated to dryness and the residues tested for radioactivity. Since the radioactivities were very low, further isolation of individual amino acids was not attempted.

### *Analysis of Urine*

The urine, collected at specified intervals, was made up each time to 25 ml with water. The total radioactivity was determined by evaporating a suitable aliquot (e.g., 0.1 ml) of the solution to dryness on an aluminum disk and measuring the activity of this sample.

*Urea*—Urea could be isolated conveniently from the urine by making use of the xanthydril precipitation method of Allen and Luck (6). To a 2 ml sample of the urine solution were added 3 ml of water and 5 ml of glacial acetic acid. Then 0.5 ml of 10 per cent xanthydril (in methanol) was added, and after an hour, the resulting precipitate of crystalline dixanthydril urea was collected on filter paper. The crystals were washed first with 50 per cent acetic acid and then with methanol, dried, and counted for radioactivity.

*L-Tyrosine*—A weighed sample (approximately 20 mg) of pure L-tyrosine carrier was added to a 2 ml aliquot of the urine solution. After heating to dissolve the tyrosine, the solution was adjusted to pH 5.7, concentrated,

and cooled to 5°, whereupon the tyrosine crystallized out. The latter was recrystallized three times from boiling water. The radioactivity values were corrected for the loss of tyrosine during the recrystallizations.

*DL-Tyrosine*—The same procedure as that for L-tyrosine was followed, except that a DL carrier was employed.

*Hippuric Acid*—The procedure was the same as that for L-tyrosine, except that the hippuric acid was crystallized out by acidifying the solution with hydrochloric acid.

*Creatinine*—A weighed quantity (approximately 20 mg) of creatinine was added to 2 ml of the urine solution. The creatinine was then recovered and purified as described by Bloch and Schoenheimer (7).

*Ketone Bodies*—Two methods employing carriers were used for isolation. The first consisted of the addition of about 50 mg of acetoacetic acid to 5 ml of urine solution, and then recovery of this acid as the 2,4-dinitrophenylhydrazine derivative. The second method involved the addition of about 10 mg of acetone to 5 ml of urine solution and recovery of the acetone as the mercury sulfate complex, following treatment with Denigès reagent and potassium dichromate according to the Van Slyke method. However, since tyrosine also yields a precipitate upon oxidation with dichromate, the urine was first freed of tyrosine<sup>3</sup> by the repeated addition and removal of 50 mg portions of the DL-tyrosine carrier. Six repetitions of this process were required to remove all of the radioactive tyrosine.

## RESULTS AND DISCUSSION

### *Constituents of Tissues*

*Incorporation of C<sup>14</sup> into Proteins*—The relative concentrations and total contents of radioactive carbon in the proteins of the various organs of normal and tumor-bearing rats following the administration of tyrosine\* are given in Table I.

In the left half of Table I it is seen that, in the course of the 6 hour periods, the intestinal mucosa attained the highest concentrations of C<sup>14</sup>. Since the tyrosine was given intravenously, this finding is not complicated by the factor of absorption. The high rate of protein synthesis by the intestinal mucosa has been observed recently in experiments with labeled methionine (8). The kidneys and plasma had the next highest activities, followed by the liver. The testes, carcass, and brain had lower C<sup>14</sup> concentrations, while muscle was still less active. The red blood corpuscles were the least active of all tissues examined, since their concentrations of C<sup>14</sup> were generally too low to be detected by the Geiger counter.<sup>3</sup> The high activity

<sup>3</sup> The incubation *in vitro* of rat blood with tyrosine\* likewise resulted in no appreciable uptake of C<sup>14</sup> by either red cells or plasma proteins.

of the tumor protein is significant. In general, the normal and sarcoma tissues exhibited rather similar patterns.

The results for the 3 and 5 day periods resemble those of the 6 hour experiments, except that the  $C^{14}$  concentrations were progressively reduced. In addition, the results for the 5 day period indicate a much more uniform redistribution of the  $C^{14}$  and a tendency for the different tissues ultimately to reach a common  $C^{14}$  concentration.

TABLE I  
*Incorporation of  $C^{14}$  into Proteins Following Administration of Radioactive Tyrosine to Rats*

Organ or tissue	Activity per gm. protein as per cent of administered dose					Total activity in protein of whole organ as per cent of administered dose*				
	Normal rat			Rat with sarcoma		Normal rat			Rat with sarcoma	
	6 hrs	3 days	5 days	6 hrs	3 days	6 hrs	3 days	5 days	6 hrs	3 days
Intestinal mucosa	7.0	2.3	0.4	7.3	1.5	1.90	0.55	0.08	1.05	0.40
Kidney	5.3	2.8	1.2	4.5	2.8	1.80	0.80	1.05	1.25	1.10
Plasma	4.0	2.8		4.3	1.5	0.90	0.50		0.85	0.30
Spleen	†	1.5	0.5	†	1.3	†	0.16	0.05	†	0.50†
Liver	2.3	1.5	0.7	2.0	0.9	3.20	2.40	0.95	3.55†	2.10†
Testes	1.3	1.2	0.8	0.8	0.7	0.30	0.20	0.15	0.20	0.13
Carcass	0.7	0.5	0.5	0.3	0.3	15.5	8.0	8.0	10.3	6.8
Brain	0.5	0.6	0.5	0.5	0.3	0.12	0.12	0.08	0.11	0.06
Muscle	0.2	0.1	0.2	0.1	0.1	†	†	†	†	†
Red blood cells	0.0	0.0	0.1	0.0	0.0	0.00	0.00	0.10	0.00	0.00
Tumor				4.0	1.4				8.3	3.95

\* The product of the activity per gm. of protein and the weight (in gm.) of the protein of the entire organ.

† Included in the carcass value.

‡ This organ was somewhat enlarged.

Because of the probable relationship of tyrosine to adrenalin and thyroxine, it was of interest to measure the specific radioactivity of the adrenal and thyroid proteins. These activities, however, were only of moderate magnitude, each about equal to that of the testicular protein. The non-protein fractions of the two glands had very low activities.

The right half of Table I affords a comparison of the total  $C^{14}$  uptakes by the different organs. It may be noted that the tumor tissue of the sarcoma-bearing rats accounted for one-third and one-fourth of the total  $C^{14}$  incorporated into body proteins for the 6 hour and 3 day periods, although the tumors comprised only 9 and 11 per cent, respectively, of the total weights of the animals. The generally lower  $C^{14}$  content of the different organs in the sarcoma-bearing rats, as compared to the normal animals,

appears to be a consequence of both the smaller size of these organs (liver and spleen excepted) and their slightly lower  $C^{14}$  concentration

$C^{14}$  Content of Isolated Tyrosine—Table II gives the distribution of tyrosine<sup>a</sup> in the various organs of the normal and the sarcoma-bearing rats for the 6 hour experiments. Qualitatively similar data (not included) were obtained for the 3 day periods. The relative  $C^{14}$  concentrations in the tyrosine preparations parallel fairly well the activities of the proteins in Table I, while their total  $C^{14}$  content accounts for about one-third of the administered dose. As will be indicated subsequently, the tyrosine accounted for almost all of the radioactivity of the tissue proteins. By contrast,

TABLE II  
*C<sup>14</sup> Content of Tyrosine Isolated from Tissue Proteins*

	Activity per mg. tyrosine as per cent of administered dose		Total activity in tyrosine of whole organ as per cent of administered dose <sup>a</sup>	
	Normal rat	Rat with sarcoma	Normal rat	Rat with sarcoma
Intestinal mucosa	0.235	0.160	2.1	0.81
Kidney	0.105	0.09	1.3	1.05
Plasma	0.08	0.10	0.60	0.65
Liver	0.07	0.045	3.7	3.45
Carcass	0.025	0.02	25.0	19.0
Testes	0.025	0.02	0.2	0.16
Brain	0.02	0.01	0.2	0.07
Muscle	0.015	0.01	1.1	0.36
Tumor		0.10		9.75

<sup>a</sup> Product of the activity per mg. of tyrosine  $\times$  per cent tyrosine in protein  $\times$  total weight of protein. The percentage of tyrosine was determined by colorimetric analysis (5) on material from other rats.

Schoenheimer, Ratner, and Rittenberg (1) estimated that only a fourth of the  $N^{15}$  incorporated into proteins was due specifically to tyrosine, following the feeding of this isotopically labeled amino acid to rats.

*Dicarboxylic Amino Acids*—Very small concentrations of  $C^{14}$  were detected in the dicarboxylic acid fractions of the more active tissues (Table III). If the total dicarboxylic amino acid content of the organs is taken into account, it may be estimated that the  $C^{14}$  in these amino acids amounts to less than 1 per cent of the administered doses. Still lower values were obtained for the 3 day periods. The finding that  $C^{14}$  appears in dicarboxylic amino acids after feeding tyrosine is in agreement with a separate experiment (not recorded in the present paper) in which a considerable fraction of the  $C^{14}$  was found in the respired carbon dioxide after the administration of

labeled tyrosine to a rat Delluva and Wilson (9) have found recently that the injection of isotopic bicarbonate results in its incorporation into both glutamic and aspartic acids, and have discussed the possible pathway for this transformation Schoenheimer and coworkers (1) observed that moderate quantities of isotopic dicarboxylic amino acids were formed from tyrosine containing  $N^{15}$ , but concluded that this effect was chiefly one of amino nitrogen transfer

*Arginine*—None of the diflavianates, including those from liver proteins, had detectable concentrations of  $C^{14}$  However, small but definite  $C^{14}$  concentrations were found in the urea isolated from urine (Table V) Schoenheimer and coworkers (1) found  $N^{15}$  in both the guanidine moiety of liver arginine and in urinary urea following the feeding of isotopic tyrosine, in agreement with the Krebs-Hanseleit theory Our inability to detect  $C^{14}$

TABLE III  
*C<sup>14</sup> Content of Dicarboxylic Amino Acids*

Tissue	Activity as per cent of administered dose per mg barium salt $\times 1000$	
	Normal rat (6 hrs )	Rat with sarcoma (6 hrs )
Intestinal mucosa	1.2	0.8
Kidney	0.8	0.5
Liver	0.3	0.3
Plasma	0.2	0.3
Testes	0.2	0.1
Tumor		0.7

in liver arginine is probably a consequence of too great a dilution of the isotopic form of this amino acid by the normal arginine of the liver

*Residual Amino Acids*—These fractions were low in activity and comprised about 0.5 per cent of the administered dose in each animal However, appreciable quantities of radioactive neutral amino acids may have been coprecipitated with the crude tyrosine and thus lost to the residual fractions

### *Constituents of Urine*

Table IV indicates that the rates of urinary excretion of  $C^{14}$  were similar for the normal and the sarcoma-bearing rats during a 3 day period Following the initial rapid excretion, the subsequent elimination of radioactive substances fell to a low rate

The initial rapid excretion probably reflects the elimination of the incomplete oxidation products from the catabolism of the free tyrosine Subse-

quently there is a much slower excretion of the products of catabolism of the tyrosine incorporated into the tissues

The substances that were isolated, listed in Table V, account for only a fraction of the total radioactivities of the urine samples

*Tyrosine*—The fact that the DL carrier accounted for approximately twice as much  $C^{14}$  as the L carrier suggests that approximately equal proportions

TABLE IV

*Rate of Urinary Excretion of  $C^{14}$  after Administration of Radioactive Tyrosine*

Time after administration <i>days</i>	Per cent of administered radioactivity in urine	
	Normal rat	Rat with sarcoma
$\frac{1}{2}$	23.5	27.0
1	1.6	2.4
2	1.0	1.3
3	0.5	0.8

TABLE V

*Distribution of  $C^{14}$  in Urine Collected 6 Hours after Administration of Radioactive Tyrosine*

Constituent	Activity in per cent of administered dose	
	Normal rat	Rat with sarcoma
Total constituents	18.5	15.5
L-Tyrosine	2.45	1.8
DL-Tyrosine	4.0	3.4
Urea	0.8	0.5*
Acetoacetic acid (dinitrophenylhydrazine)	1.6	
Ketone bodies (Deniges ppt.)	1.3	
Hippuric acid	0.5	0.2
Creatinine	0.1	0.1

\* The value 0.15 was obtained when urea was decomposed by urease and the  $CO_2$  trapped in  $Ba(OH)_2$ .

of L- and D-tyrosine were present in the urine. In experiments with cats (10) and humans (11), in which relatively large amounts of DL-tyrosine were fed, the urine was reported to contain more of the D than of the L form.

*Urea*—The finding of  $C^{14}$  in urea agrees with the experiment, already cited, in which the carbon dioxide of the expired air contained  $C^{14}$ .

*Ketone Bodies*—In agreement with the view of Wakeman and Dakin (12) and Edson (13) that ketone bodies may be formed from tyrosine, a considerable  $C^{14}$  concentration was found in the ketones isolated from the

urine Butts, Dunn, and Hallman (14) failed to find ketone bodies in the urine of rats after feeding ordinary DL-tyrosine, although subsequently Butts, Sinnhuber, and Dunn (15) found L-tyrosine to be ketogenic

*Hippuric Acid and Creatinine*—The finding of  $C^{14}$  in both these compounds makes it highly probable that small amounts of glycine were formed from the tyrosine. Inasmuch as the formation of glycine from acetic acid has been observed, the following sequence appears plausible: tyrosine  $\rightarrow$  acetoacetate  $\rightarrow$  acetate  $\rightarrow$  glycine

#### SUMMARY

Tyrosine labeled with radioactive carbon,  $C^{14}$ , was used to investigate the metabolism of this amino acid in normal and in tumor-bearing rats.  $C^{14}$  was rapidly incorporated into the proteins of the various organs of the animals, following the administration of the tyrosine. Intestinal mucosa, kidney, and plasma had the highest  $C^{14}$  concentrations, followed by liver and spleen. Lower concentrations of the isotope were found in testes, brain, and muscle. The tumor proteins had high activity and accounted for sizable fractions of the total protein-bound  $C^{14}$ .

Amino acid isolations showed that almost all of the radioactivity of the proteins was due to tyrosine itself. Low  $C^{14}$  concentrations were found in the dicarboxylic acids.

Analysis of the urinary constituents indicated the presence of  $C^{14}$  in tyrosine, urea, creatinine, hippuric acid, and ketone bodies.

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# STUDIES IN PROTEIN METABOLISM WITH COMPOUNDS LABELED WITH RADIOACTIVE CARBON

## II THE METABOLISM OF GLYCINE IN THE RAT\*

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Glycine labeled with heavy nitrogen ( $N^{15}$ ) has been employed by Ratner, Rittenberg, Keston, and Schoenheimer (1) and by Shemin and Rittenberg (2) to study the metabolism of this amino acid in rats. The  $N^{15}$  concentrations in the proteins after the feeding of labeled glycine were indicative of the rates of protein regeneration in the various organs of the animals. From hydrolysates of the proteins, different  $N^{15}$ -containing amino acids were isolated. The presence of  $N^{15}$  in these compounds reflected amino nitrogen transfer.

Other papers have dealt with the rôle of glycine as a precursor of glutathione (3), protoporphyrin (4), and uric acid (5, 6).

The present paper is concerned with the fate of radioactive glycine (glycine\*), labeled on the carboxyl carbon with  $C^{14}$ , in rats. With this compound, the relative rates of incorporation of  $C^{14}$  into the proteins of the different organs, and also the retention of  $C^{14}$  by these proteins, were determined. The utilization of the carboxyl carbon of the glycine\* for the formation of other amino acids was studied, and the distribution of  $C^{14}$  among certain important urinary constituents was determined.

### EXPERIMENTAL

The glycine\* was synthesized by Ostwald (7). Its activity corresponded to approximately 25,000 counts per mg. per minute with the Geiger counter. The technique of radioactivity measurements was described in Paper I of this series (8).

*Incorporation of  $C^{14}$  into Proteins*—Five male rats, each weighing approximately 180 gm., were fasted overnight and then given a single dose of 25 mg. of glycine\* in 1 ml. of water by a specified route. Then the animals were sacrificed after different intervals. The protein fractions from the different organs were prepared and their radioactivity determined as previously described (8).

*Analysis of Urine*—The distribution of  $C^{14}$  among certain urinary con-

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stituents was determined as already described (8). In the case of the ketone bodies, the Van Slyke method, in which Denigès reagent was employed, was applied directly to a fraction of the urine to which the acetone carrier had been added.

*Isolation of Amino Acids*—In order to attain a greater degree of incorporation of  $C^{14}$  into the tissue proteins, five 10 mg doses of glycine\* were given (by stomach tube) at about 10 hour intervals to a 200 gm male rat. 10 hours after the last administration the animal was killed. The protein fractions, prepared from the following organs were pooled: liver, kidney, lungs, intestines, spleen, testes, and plasma. This material, weighing 4.1 gm, was hydrolyzed by autoclaving it with 50 ml of 4 N hydrochloric acid for 18 hours at 15 pounds pressure. The hydrolysate was decolorized with carbon, evaporated to dryness *in vacuo*, and subsequently diluted to 50 ml with water.

In analyzing the hydrolysate, we simply determined the percentages of the total  $C^{14}$  contained in the different amino acids. For this purpose, separate aliquots of the hydrolysate were employed for the isolation of individual amino acids. Non-radioactive carriers were added to the aliquots, to make possible the recovery of the labeled amino acid in question in both higher yield and in purer form.

*Dicarboxylic Amino Acids*—200 mg of L-glutamic acid and 100 mg of L-aspartic acid were added to 10 ml (one-fifth) of the hydrolysate. From the latter the mixed calcium salts of the dicarboxylic acids were prepared.

After removal of the calcium with oxalate, glutamic acid was separated by crystallization from a concentrated hydrochloric acid solution. It was then recrystallized from the same solvent. Found, N 7.85, theory, N 7.67.

Aspartic acid was precipitated from neutral solution as the copper salt. The latter was decomposed with hydrogen sulfide, the copper sulfide removed, and the free aspartic acid crystallized from aqueous alcohol solution. Found, N 10.25, theory, N 10.53.

*Arginine*—To 5 ml (one-tenth) of the hydrolysate were added 50 mg of L-arginine hydrochloride. Arginine diisocyanate was subsequently isolated, and from it arginine monohydrochloride was prepared. Found, N 26.0, theory, N 26.7.

*Glycine*—To a 1 ml aliquot of hydrolysate (pH 1) were added 15 mg of glycine and 150 mg of potassium trioxalatochromate. Then 3 volumes of ethyl alcohol were added, and the mixture shaken for 8 hours. The precipitated glycine salt was recrystallized twice from dilute hydrochloric acid by adding 2 volumes of alcohol. Found, N 2.15, theory, N 2.5 for  $(C_1(C_2O_3)_3)_6K_3(C_2H_5O_2N)_5 \cdot 3H_2O$  (9).

*Proline*—To 3 ml of hydrolysate were added 50 mg of L-proline, followed by 200 mg of ammonium rhodanilate (10) in 3 ml of methanol. The re-

sulting crystalline precipitate of proline rhodanilate was recrystallized twice from methanol by adding 0.1 N hydrochloric acid. Found, N 15.4, theory, N 16.2 for  $(C_{16}H_{14}N_6S_4Cr)(C_5H_{10}O_2N) \cdot H_2O$

*Serine*—To 5 ml of hydrolysate were added 200 mg of DL-serine. Then 0.6 ml of methyl cellosolve and 800 mg of *p*-hydroxy azobenzene-*p*-sulfonic acid were added. The resulting serine salt was purified by the method of Stein and coworkers (11). Subsequently the salt was decomposed with barium acetate, and serine was isolated and recrystallized from aqueous alcohol. Found, N 12.9, theory, N 13.3

TABLE I

*Relative Rates of  $C^{14}$  Uptake and Turnover by Proteins of Different Organs of Rats*

Organ	Activity in per cent of administered $C^{14}$ found per gm protein after				
	$\frac{1}{2}$ hr *	6 hrs *	18 hrs	3 days	5 days
Intestinal mucosa	0.3	3.7	3.25	1.35	0.95
Bone marrow	0.2	2.15	2.2	1.4	0.9
Liver	0.25	2.05	1.35	1.2	0.85
Kidney	0.15	1.95	1.3	1.1	0.95
Plasma	0.05	1.8	1.6	0.95	0.75
Spleen	0.05	1.45	1.25	0.8	0.6
Lung	0.15	1.25	1.1	0.85	0.65
Testes	0.05	0.5	0.5	0.45	0.4
Muscle	0.0	0.15	0.2	0.2	0.2
Red blood cells	0.0	0.15	0.15	0.2	0.25
Brain	0.0	0.1	0.15	0.1	0.1

\* The  $\frac{1}{2}$  and 6 hour values are for glycine\* given intravenously, and hence are not strictly comparable with those for the longer times, which correspond to administration by stomach tube

*Tyrosine*—This amino acid was isolated and purified as previously described (8)

#### RESULTS AND DISCUSSION

*Incorporation of  $C^{14}$  into Proteins*—The data in Table I indicate that following the administration of a single dose of glycine\* the  $C^{14}$  concentration in the proteins of most organs reached a peak within approximately 6 to 18 hours. Subsequently the values approached a common lower level. While the  $C^{14}$  levels in the proteins were very low in the  $\frac{1}{2}$  hour experiments, the corresponding non-protein filtrates were found to have very high radioactivity. In the 3 and 5 day experiments, the non-protein filtrates had negligible activity.

The relative activities of the different organs conform fairly well to the

pattern observed in studies with labeled methionine (12) and tyrosine (8). Proteins of the intestinal mucosa showed the greatest ability to accumulate  $C^{14}$ . Other active tissues were bone marrow, liver, kidney, plasma, spleen, and lung. Least active were muscle, red blood cells, and brain. These results are qualitatively similar to those of Ratner and coworkers (1), who found high  $N^{15}$  concentrations in proteins of liver, intestines, and serum, but low levels in muscle, brain, and erythrocytes, following the feeding of  $N^{15}$ -labeled glycine.

Shemin and Rittenberg (2) likewise found that proteins which started above the average isotope level steadily decreased in  $N^{15}$  concentration, while the carcass slowly increased with time. They estimated the half life

TABLE II  
*Distribution of  $C^{14}$  among Amino Acids of Tissue Proteins*

Amino acid	$C^{14}$ content in per cent of total $C^{14}$ in hydrolysate*
Glycine	60†
Glutamic acid	12
Aspartic acid	5
Arginine	3
Serine	0
Proline	0
Tyrosine	0

\* Inasmuch as several doses of glycine\* were administered and pooled tissue proteins hydrolyzed, it was not feasible to express the activities of the amino acids in terms of the administered glycine\*, as was done in Paper I (8).

† In a separate experiment in which the glycine\* was isolated with the aid of excess carrier, a value of 68 per cent was obtained.

of  $N^{15}$  in the liver proteins to be 6.5 days. While our data are not sufficiently extensive to permit an accurate calculation, we estimate the half life of  $C^{14}$  to be approximately 5 days in the proteins of the more active internal organs, including liver.

From the weights of the proteins of the different organs, it was calculated that the total  $C^{14}$  incorporated comprised about 8 per cent of the administered dose in either the 6 or 18 hour experiments. Less than 4 per cent of the  $C^{14}$  appeared in the urine in 18 hours. However, about 50 per cent of the administered  $C^{14}$  was recovered in the carbon dioxide of the expired air during this interval. The same result was obtained by Olsen, Hemingway, and Nier (13) when  $C^{13}$ -labeled glycine was fed to mice.

$C^{14}$  Content of Amino Acids—Table II indicates that most of the radioactivity in the tissue proteins was accounted for as glycine. The lower concentration of  $C^{14}$  in glutamic and aspartic acids was derived from

glycine by mechanisms still unknown. No radioactivity could be detected in the serine, proline, or tyrosine. While the precursors of serine and proline are unknown (14), the present results cast doubt on the supposition that the glycine molecule is a precursor of either of these amino acids in the rat. Very recently Ehrensverd and coworkers have found that labeled glycine may give rise to isotopic proline and serine in yeast (15).

Shemin and Rittenberg (2) found a high isotope concentration in glycine and lower concentrations in glutamic acid and arginine after feeding  $N^{15}$ -labeled glycine. The tyrosine isolated by them also contained isotope, reflecting amino nitrogen transfer.

*Distribution of  $C^{14}$  in Urine*—Table III indicates that hippuric acid and free glycine together account for about two-fifths of the total  $C^{14}$  of the

TABLE III

*Distribution of  $C^{14}$  among Urinary Constituents*

The urine was collected over an 18 hour period

Compound	$C^{14}$ content in per cent of administered dose
Total constituents	3.7
Urea	0.8
Hippuric acid	1.0
Glycine	0.5
Creatinine	0.2
Ketone bodies	0.1

urine. Creatinine had a smaller concentration of  $C^{14}$ , very likely contained in the glycine component.

One-fifth of the urinary  $C^{14}$  was present in urea. This  $C^{14}$  is probably derived chiefly from the liver arginine. The feeding of  $N^{15}$ -labeled glycine was shown (2) to result in a concentration of  $N^{15}$  in urinary urea very nearly the same as that in the amidine group of liver arginine.

The very low radioactivity in the ketone bodies is in accord with the view that glycine is not ketogenic. By contrast, the administration of tyrosine\* led to a much higher  $C^{14}$  concentration in the urinary ketones (8).

## SUMMARY

Glycine, labeled in the carboxyl group with radioactive carbon,  $C^{14}$ , was used to study the metabolism of this amino acid in rats. The  $C^{14}$  concentrations in the proteins of the different body organs were determined for varying time intervals following the administration of single doses of the labeled glycine to the animals. In most organs the radioactivity reached a peak after about 6 to 18 hours. Thereafter the  $C^{14}$  concentrations slowly

approached a common lower level. The more active organs were intestines, bone marrow, liver, kidney, plasma, spleen, and lung. Muscle, red blood cells, and brain were least active in  $C^{14}$  uptake.

Approximately 60 per cent of the  $C^{14}$  in the hydrolysate of the proteins was accounted for as glycine. Much smaller  $C^{14}$  concentrations were found in glutamic acid, aspartic acid, and arginine. Tyrosine, serine, and proline were inactive.

18 hours after the administration of radioglycine, about 50 per cent of its  $C^{14}$  was eliminated as respiratory carbon dioxide, while only 4 per cent appeared in the urine.

Urea, hippuric acid, and free glycine accounted for most of the urinary  $C^{14}$ , while low radioactivities were found in creatinine and in the ketone bodies.

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# THE PREPARATION OF SODIUM ACETATE LABELED WITH RADIOACTIVE CARBON IN THE METHYL GROUP\*

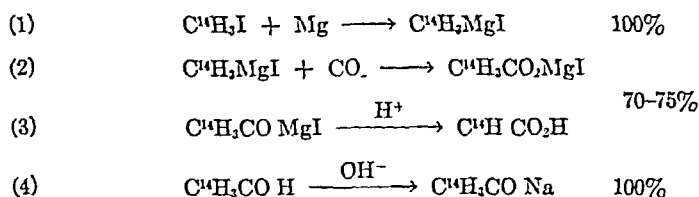
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The purpose of this paper is to describe the preparation of sodium acetate from methyl iodide<sup>1</sup> on a 15 to 25 mm scale by carbonating methyl magnesium iodide

The several steps of the preparation and yields are as follows



Steps (1) and (2) were carried out in an evacuated closed system (Fig 1) The 150 ml conical reaction flask, *D*, containing 50 ml of dry ether and 0.5 gm of magnesium turnings, was chilled with liquid nitrogen and 1 ml (2.28 gm) of methyl iodide distilled in from storage vessel *B*. The reaction vessel was closed off and the ether refluxed 1 hour. The reaction flask was then cooled to  $-20^\circ$  and carbon dioxide, that had been dried by passing through a spiral immersed in dry ice-acetone and freed of oxygen and nitrogen by condensing with liquid air and evacuating at low pressures, was added from bulb *J* until a pressure of about 30 cm was maintained in the system. Stirring was then continued for 10 minutes. The reaction vessel was removed from the line and opened in the hood and the cold ( $-20^\circ$  to  $-50^\circ$ ) Grignard complex decomposed with 15 ml of 6 N sulfuric acid. After decomposition, an additional 35 ml of water were added. 5 gm of silver sulfate were added to precipitate the iodide present. The ether was distilled off and the acetic acid was steam-distilled from the reaction mixture with about 300 ml of water. This distillate was exactly neutralized with 1 N sodium hydroxide solution with a glass electrode, evaporated to a small volume, filtered, evaporated to dryness, and dried *in vacuo* at  $10^{-3}$

\* This paper is based on work performed under contract No W-7405-Eng-48 of the Atomic Energy Commission with the University of California

<sup>1</sup> Tolbert, B M, *J Am Chem Soc*, 69, 1529 (1947)



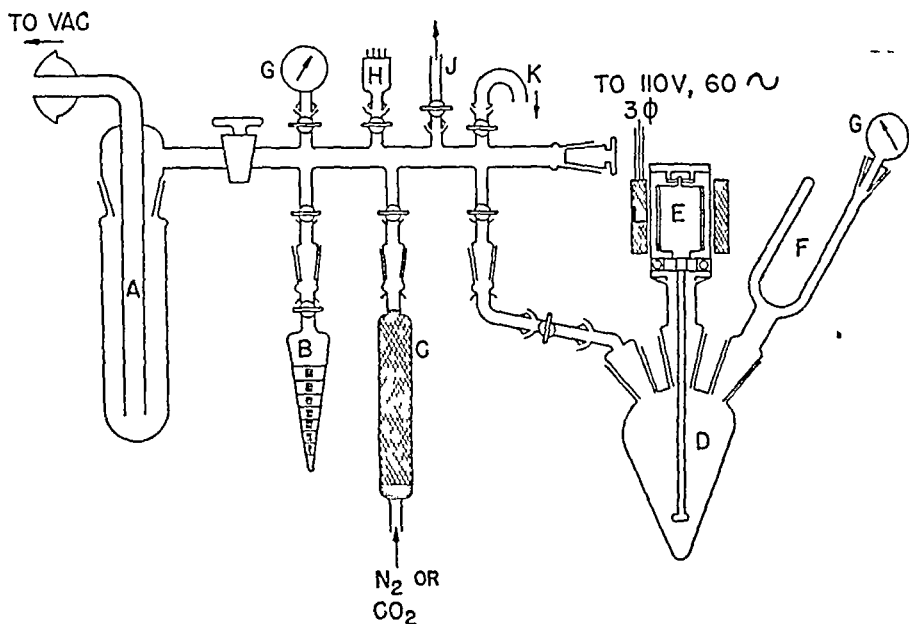


FIG 1 Grignard preparation and carbonation unit *A*, large liquid air trap, *B*, methyl iodide storage vessel, *C*, drying tube, *D*, Grignard flask, *E*, induction stirrer, *F*, dry ice-acetone reflux condenser, *G*, Bourdon type vacuum gages, *H*, thermocouple type vacuum gage, *J*, to  $\text{CO}_2$  storage bulb, *K*, mercury manometer, *L*, Selsyn generator stator Note that the manifold may be readily removed for cleaning

mm pressure The yield of white anhydrous sodium acetate was 70 to 75 per cent, the titration and weighings agreeing within a few tenths per cent

In several test runs with inactive methyl iodide, titration of the Grignard solution showed the yield to be 98 to 100 per cent in this step The silver sulfate is necessary to precipitate the iodide from the reaction mixture, if it is not added, free iodine will distil over and the product will be contaminated with iodine compounds

#### SUMMARY

Sodium acetate labeled with  $\text{C}^{14}$  in the methyl group has been prepared on a 15 to 25 mm scale in 70 to 75 per cent yield by carbonating labeled methyl magnesium iodide in a vacuum system

# SYNTHESIS OF CHLOROACETIC ACID AND GLYCINE LABELED WITH RADIOACTIVE CARBON IN THE CARBOXYL GROUP\*

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(Received for publication, October 15, 1947)

In order to investigate certain aspects of protein metabolism, it was desired to prepare glycine labeled with radioactive carbon. The method of preparation was chosen with the object of obtaining the highest possible yields, and techniques suitable for handling small amounts of material were used throughout. The reactions employed and the yields obtained were as follows.

The synthesis involves three steps, namely, conversion of sodium acetate to acetic acid (yield, 98 per cent), chlorination to monochloroacetic acid with  $\text{PCl}_5$  in the presence of phosphorus and iodine (yield, 70 to 90 per cent), and amination with ammonia in the presence of ammonium carbonate (yield, 80 to 90 per cent).

## EXPERIMENTAL

*Chloroacetic Acid*—A platinum boat charged with 1.4081 gm of carboxyl-labeled anhydrous sodium acetate (1)<sup>1</sup> was placed in a horizontal glass tube and dried *in vacuo* at  $3 \times 10^{-2}$  mm of Hg for 24 hours. The tube was then connected to a train of three traps, each of which was cooled with an isopropyl alcohol-dry ice mixture, the last trap was protected with a calcium chloride tube. Gaseous hydrogen chloride, dried with concentrated sulfuric acid, drierite, and anhydrous aluminum chloride, was passed slowly through the train. The tube was gradually heated and the liberated acetic acid distilled into the traps. When the reaction was complete, the three traps were connected to the vacuum line and the contents were distilled into a small reaction vessel (Fig. 1), which was cooled with liquid nitrogen. The product contained 10 to 15 per cent water and a consider-

\* This work was done under the auspices of the Atomic Energy Commission, under contract No. W-7405 Eng-48 with the University of California.

† Supported by a grant to Professor D. M. Greenberg, University of California, from the American Cancer Society (recommended by the Committee on Growth of the National Research Council).

<sup>1</sup> The author wishes to thank R. M. Lemmon for the preparation of the carboxyl-labeled sodium acetate.

able amount of gaseous hydrogen chloride. The reaction vessel, protected by a low temperature condenser cooled with isopropyl alcohol-dry ice and fitted with a calcium chloride tube, was warmed to room temperature. 0.65 gm of acetic anhydride was added and the mixture was refluxed for  $\frac{1}{2}$  hour to remove the water. A mixture of 0.02 gm of iodine, 0.04 gm of phosphorus, and 0.68 gm of phosphorus pentachloride was added (2), and dry chlorine was passed through the system at reflux temperature for  $2\frac{1}{2}$  hours (see Fig. 1).

After the chlorination was completed, all of the material in the condenser and in the gas inlet tube was distilled *in vacuo* back into the reaction vessel, which was cooled in liquid nitrogen. The chloroacetic acid was then

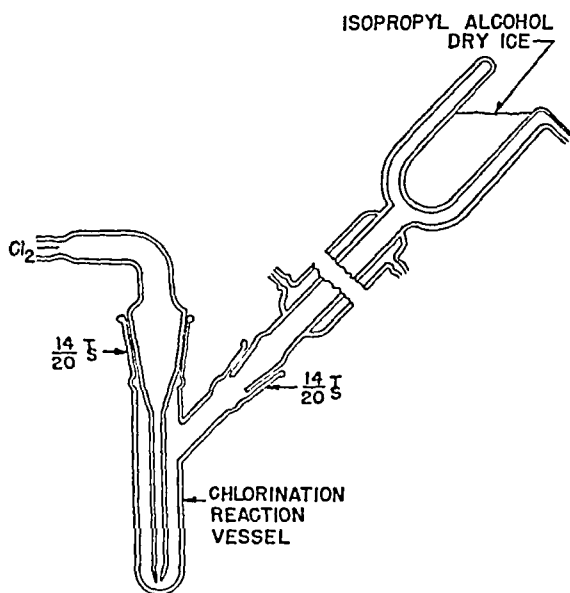


FIG. 1. Reaction vessel for chlorination.

purified by fractional sublimation *in vacuo* onto a cold finger condenser filled with powdered dry ice.

The yield of pure product was 1.52 gm, m.p.  $60^\circ$ , which is 67 per cent based on anhydrous sodium acetate. Previous runs with inactive material had given yields of 85 to 90 per cent.

The sodium acetate used had a specific activity (1, 3) of  $1.6 \times 10^5$  counts per minute per mg. The total activity was 650 microcuries. The chloroacetic acid obtained had a specific activity of  $5 \times 10^4$  counts per minute per mg and a total activity of 440 microcuries. The activity recovered from residues and washings was 100 microcuries.

**Glycine**—Glycine labeled with  $C^{13}$  has been prepared by several investigators (1-6). Glycine labeled with  $C^{14}$  has been described by Guin and Deluca (7).

A mixture of 3.2 gm of powdered ammonium carbonate, 10 ml of concentrated ammonia, and 4 ml of water was heated in a small three-neck flask, which was fitted through ground glass joints to a pressure-equalized dropping funnel, a Liebig condenser, and a thermometer. After the salt had dissolved, 1.014 gm of carboxyl-labeled chloroacetic acid in 3 ml of water were added dropwise through the dropping funnel at such a rate that the temperature of the solution did not rise above 60°. The mixture was held at 60° for 6 hours and was then allowed to stand for 12 hours at room temperature. The solution was then concentrated until its temperature reached 112°. The distillate showed only very slight radioactivity. The yellowish solution was cooled to 70°, and 15 ml of absolute methanol were added slowly with agitation. The mixture was cooled in a refrigerator for 1 hour. The precipitate was filtered and washed with methanol and ether (8, 9).

The yield of pure white crystals, which showed no trace of chloride ion, was 0.54 gm or 70 per cent (m.p. 225°, with decomposition). C 32.02, H 6.78, calculated, C 32.02, H 6.78. Upon concentration, the mother liquor gave 0.08 gm of glycine which increased the yield to 0.62 gm or 79 per cent, based on chloroacetic acid.

The chloroacetic acid used had a total activity of 299 microcuries. The glycine had a specific activity of  $6.3 \times 10^4$  counts per minute per mg and a total activity of 229 microcuries. From distillates and mother liquors, 50.4 microcuries were recovered.

#### SUMMARY

Carboxyl-labeled chloroacetic acid was prepared from sodium acetate labeled in the carboxyl position with  $C^{14}$ . Carboxyl-labeled glycine with a specific activity of  $6.3 \times 10^4$  counts per minute per mg was prepared in turn from the chloroacetic acid. The over-all yield of glycine was 42 per cent, based on sodium acetate.

The author wishes to thank Professor M. Calvin for his advice in this work.

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# THE INHIBITION OF PYRUVIC OXIDASE BY PROTOANEMONIN\*

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(Received for publication, December 3, 1947)

During the study of the biological action of protoanemonin (1, 2) it was found that this substance could inhibit the enzymatic oxidation of pyruvic acid. *Proteus vulgaris* or the cell-free pyruvic oxidase prepared from it (3) was used to carry out the oxidation of the pyruvic acid.

The addition of a solution of protoanemonin to a washed suspension of *Proteus vulgaris*, with pyruvic acid as the substrate, resulted in a marked reduction in oxygen uptake. Protoanemonin did not inhibit the action of the cell-free pyruvic oxidase under the same conditions. In searching for the cause of this discrepancy a further study was made of the pyruvic oxidase, since it presented a simpler system than the intact organism.

Pyruvic oxidase consists of a protein, diphosphothiamine as coenzyme, and a bivalent metal such as magnesium, the naturally occurring metal, or manganese. The coenzyme is easily removed from the enzyme by precipitation with acetic acid at pH 4.3, leaving the protein metal complex (3). There is, however, difficulty in completely separating the protein from its metal. The protein preparations used in these experiments almost certainly contained some of the metal, since the addition of diphosphothiamine to the protein preparations yielded solutions with some enzymatic activity. Addition of manganese sulfate solution, however, resulted in an increase in activity.

Incubation of the protein moiety with protoanemonin for about  $\frac{1}{2}$  hour prior to the addition of the remainder of the enzyme system inhibited the enzyme so that it was only capable of 20 per cent or less of its normal oxygen consumption. Treatment of the protein with diphosphothiamine or diphosphothiamine and manganese before the addition of the protoanemonin resulted in an active enzyme system. Manganese sulfate alone could not protect the protein from the partially inactivating action of protoanemonin.

In order to gain further information about this reaction the effect of other inhibitors was studied. If iodoacetic acid or *p*-chloromercuribenzoic acid was allowed to interact with the protein, it was no longer capable of functioning as part of the pyruvic oxidase system. However, the above

\* Conducted with the aid of grants from the John and Mary R. Markle Foundation and The Squibb Institute for Medical Research.

substances failed to inhibit the action of the protein-coenzyme complex. Sodium tetrathionate also destroyed the protein, but the coenzyme gave only partial protection from the action of this reagent. Sodium fluoride failed to inhibit the enzyme under any circumstances, in confirmation of Stumpf's findings.

While the cell-free enzyme oxidizes pyruvate to acetate, the intact cells oxidize acetate as well as pyruvate. The oxidation of acetate by the intact cells proceeds at a slower rate than the oxidation of pyruvate. It is of interest that acetate oxidation is also inhibited by protoanemonin (Table I).

TABLE I

*Effect of Protoanemonin on Oxidation of Pyruvate and Acetate by Proteus vulgaris*

Reagent	Oxygen uptake of control	Oxygen uptake after reagent
		Pyruvate substrate, 0.2 M
	<i>c mm per 20 min</i>	<i>c mm per 20 min</i>
Diphosphothiamine* + manganese*	48	45
“ + “ + protoanemonin, 0.003 M	45	19
Protoanemonin, 0.003 M	48	13
“ 0.017 “	36	9
		Acetate substrate, 0.2 M
“ 0.017 “	24	0

\* Diphosphothiamine = 0.5 ml of 0.1 per cent diphosphothiamine, manganese = 0.3 ml of 0.1 per cent manganese sulfate

#### DISCUSSION

There are indications that unsaturated lactones, such as protoanemonin, can react with sulfhydryl groups (4), and iodoacetic acid, *p*-chloromercuribenzoic acid, and sodium tetrathionate (5, 6) are thought to react almost exclusively with this type of protein grouping. It appears, therefore, that this pyruvic oxidase possesses essential sulfhydryl groups, this is in accord with Barion's observation on a pyruvic oxidase obtained from another source (7). Furthermore, this view is supported by the ability of BAL to reverse the inhibition by these agents.

If the sulfhydryl groups are involved in the above reactions then the metal is probably not linked to the protein through the sulfhydryl groups, for the protein preparations used contained some metal and even the addition of an excess of manganese sulfate solution failed to afford protection. However, diphosphothiamine does protect the enzyme and consequently

may be bound directly to the protein SH groups, possibly as a thiophosphoric ester. This is further supported by the fact that thiamine cannot function as the coenzyme, only the pyrophosphoric acid derivative being active. It is known that the diphosphothiamine is more easily removed from the enzyme than the metal ion, in fact, the metal is difficult to remove (3). Therefore, it is proposed that the coenzyme and the metal are each attached to the protein, but through different linkages.

It is recognized that the value of studies utilizing inhibitors is strongly dependent on the specificity of the inhibitor, and on this basis the usefulness of some sulfhydryl reagents has been questioned (8). Nevertheless, it is felt that the variety of reagents employed in these experiments, all

TABLE II  
*Effect of Inhibitors on Pyruvic Oxidase Protein*

Reagent	Oxygen uptake of control	Oxygen uptake after addition of reagent
	<i>c mm per 10 min</i>	<i>c mm per 10 min</i>
Protoanemonin, 0.003 M	22	4
" 0.0013 M	22	9
Iodoacetate, 0.003 M	29	24
" 0.017 "	29	15
p-Chloromercuribenzoate, 0.0003 M	36	0
Tetrathionate, 0.1 M	23	2
Fluoride, 0.3 M	9	10

pointing in the same direction, indicate the likelihood of the above interpretation.

#### EXPERIMENTAL

The *Proteus vulgaris* was grown on tryptose phosphate agar (Difco) in Roux bottles and washed off with 0.4 per cent saline after about 16 hours of incubation at 37°. For experiments with intact cells the organisms were washed three times with M/15 phosphate buffer and aerated for 1 hour. Pyruvic oxidase was prepared by growing the organisms as described above and then following the procedure of Stumpf (3). Different enzyme preparations varied in their rate of oxygen consumption, but otherwise no differences were observed.<sup>1</sup>

To determine the inhibitory effect of the various substances, the protein portion of the pyruvic oxidase in phosphate buffer at pH 6.2 (or 7.2 for those experiments in which p-chloromercuribenzoate was used) was incubated with the inhibitor (Table II) for ½ hour at 37°. 0.1 ml. of 0.1

<sup>1</sup> One enzyme preparation seemed to require phosphate, but all other preparations functioned without its presence. However, the results of inhibition experiments were the same with the phosphate requiring preparation as with the others.



per cent diphosphothiamine and 0.1 ml of 0.1 per cent manganese sulfate solution were then added, the volume in the cups being 2.5 ml, the substrate was 0.5 ml of 0.2 M sodium or lithium pyruvate. Oxygen uptake was measured in the Barcroft-Warburg apparatus. Separate cups prepared

TABLE III  
*Effect of Inhibitors on Whole Pyruvic Oxidase Enzyme*

Reagent	Oxygen uptake of control	Oxygen uptake after addition of reagent
	<i>c mm per 10 min</i>	<i>c mm per 10 min</i>
Protoanemonin, 0.003 M	22	22
<i>p</i> -Chloromercuribenzoate, 0.0003 M	36	28
Iodoacetate, 0.017 M	28	28
Tetrathionate, 0.1 "	23	8

TABLE IV  
*Attempted Protection from Inhibition of Pyruvic Oxidase Protein*

Inhibitor	Protecting agent	Oxygen uptake of control	Oxygen uptake after inhibitor
		<i>c mm per 10 min</i>	<i>c mm per 10 min</i>
<i>p</i> -Chloromercuribenzoate, 0.0003 M	Manganese sulfate	36	0
" 0.0003 "	Diphosphothiamine	36	27
Protoanemonin, 0.002 M	Manganese sulfate	30	13
" 0.002 "	Diphosphothiamine	30	30

TABLE V  
*Reversal by BAL of Pyruvic Oxidase Protein Inhibition*

Inhibitor added to protein	Oxygen uptake of control	Oxygen uptake after inhibitor	Oxygen uptake after inhibitor and BAL
	<i>c mm per 20 min</i>	<i>c mm per 20 min</i>	<i>c mm per 20 min</i>
Protoanemonin, 0.003 M	91	0	79
Tetrathionate, 0.1 M	91	5	63
<i>p</i> -Chloromercuribenzoate, 0.0003 M	14	0	13

in an identical manner but with buffer substituted for the inhibitor were used as controls. The data in Table III were obtained by incubating the complete enzyme with the inhibitor for  $\frac{1}{2}$  hour and then measuring the oxygen uptake.

Protection of the protein was measured by incubating the protein in buffer with the protecting agent (Table IV) for 15 minutes at 37°. After

adding the inhibitor and incubating for another  $\frac{1}{2}$  hour the remainder of the enzyme components was added and the oxygen uptake measured. Again, separate cups served as controls.

The effect of BAL was determined by incubating the pyruvic oxidase protein with the inhibitor at 37° for 30 minutes, then adding 0.3 ml of an 0.3 M solution of BAL, incubating for another 30 minutes and, finally, adding the remainder of the enzyme components (Table V).

Pyruvic acid was used in the form of its lithium or sodium salt, protoanemonin was stored in the form of a 1 or 0.5 per cent stock solution (2) and diluted when necessary. *p*-Chloromercuribenzoic acid was obtained from Dr S. Dickman and solutions prepared by dissolving it in an equivalent of sodium hydroxide solution and then diluting in water to the final concentration. The author is indebted to Dr David Green and to Merck and Company, Inc., for samples of diphosphothuamane, and to Dr A. Gilman for BAL and sodium tetrathionate.

#### SUMMARY

Protoanemonin inhibits the oxidation of pyruvic acid by intact cells of *Proteus vulgaris*, but does not inhibit this oxidation by the cell-free enzyme. However, if the protein portion of the enzyme is treated with this reagent, the enzyme is partially inactivated but the protein may be protected by the coenzyme if this is added prior to the inhibitor. Iodoacetic acid and *p*-chloromercuribenzoic acid act in an analogous fashion toward the cell-free enzyme, while fluoride does not inhibit under any conditions. Inhibition of the coenzyme-free protein by two of these substances was reversed by BAL.

The author wishes to express his thanks and gratitude to Dr B. C. Seegal and to Dr David Green.

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# ON THE SPECIFICITY OF CHICKEN PANCREAS CONJUGASE ( $\gamma$ -GLUTAMIC ACID CARBOXYPEPTIDASE)\*

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The early studies on conjugase from chicken pancreas (1, 2) did not lead to the identification of this enzyme. Subsequent progress in the elucidation of the chemical structure of pteroylglutamic acid (3) and its derivatives (4, 5) left little doubt that conjugase is one of the peptidases. Piffner *et al.* (4) classified the conjugase enzymes as carboxypeptidases after they showed that the methyl ester of pteroylheptaglutamate was not attacked by conjugase, while the free acid was attacked.

In the present paper the results of some experiments designed to determine the specificity of conjugase toward several suspected substrates are reported.

## Methods

Conjugase was prepared by a method similar to that previously described (2). The main difference was in the time of autolysis. Since we were forced to buy chicken pancreas from a distant location,<sup>1</sup> samples as received in this laboratory varied considerably in both original activity and resistance to autolysis. Only occasional samples were comparable in potency to fresh pancreas preparations. Since the autolysis of the majority of samples led to a definite decrease in activity, the optimal time of autolysis for each shipment was determined. This time, varying from 0 to 24 hours, was then used for the larger scale preparations. The decreased or, most commonly, the omitted time of autolysis resulted in a less complete extraction of conjugase and the retention of a larger amount of inert protein. The activity of most of the material thus prepared was between 20,000 and 100,000 units per mg., as determined by microbiological assay. These values are comparable to those obtained in the second stage of the preparations previously described (2). It was found that conjugase did not lose activity after several months when, after being frozen, it was stored at  $-18^{\circ}$ .

The substrates to be investigated were dissolved or suspended in 0.2 M

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<sup>1</sup> Obtained from the Chester B. Franz Company, Mammoth Spring, Arkansas.

borate buffer, pH 7.8, so that the resulting solution was 0.1 M with respect to the substrate. Borate buffer was used in preference to veronal buffer, since the latter was found to inhibit conjugase. An equal volume of conjugase solution, containing from 1 to 5 mg of protein per cc and made 0.02 M with respect to calcium chloride, was added, and the mixture was incubated for varying periods of time at 32°. Samples of 0.2 cc were withdrawn for the titration of liberated carboxyl groups (10 micromoles of substrate), samples of 0.1 cc (equivalent to 5 micromoles of substrate) were used for manometric determinations. For microbiological assays, appropriate dilutions from a 0.1 cc sample were made.

Liberated carboxyl groups were titrated in 95 per cent alcohol according to the method of Grassmann and Heyde (6). Liberated glutamic acid was determined manometrically, with either bacterial decarboxylase<sup>2</sup> (7) or squash decarboxylase<sup>3</sup> (8). None of the peptides investigated was attacked by the bacterial decarboxylase. A large majority of the squash preparations also did not attack these substrates. Only two exceptions were noted, squash decarboxylase slowly decarboxylated L-glutamyl-L-glutamic acid and carbobenzoxy-L-phenylalanyl-L-glutamic acid. Apparently these two preparations of squash decarboxylase still contained an enzyme capable of hydrolyzing the terminal glutamic acid.

#### EXPERIMENTAL

Conjugase, prepared as described above, was found to be free of any enzymes attacking glutamic acid. Incubation of varied amounts of glutamic acid (1 to 7 micromoles) with conjugase for periods ranging from 2 to 24 hours resulted in the subsequent recovery of glutamic acid within the limits of experimental error.

When the substrates<sup>4</sup>  $\alpha$ -L-glutamyl-L-glutamic acid, carbobenzoxy- $\alpha$ -L-glutamyl-L-glutamic acid, carbobenzoxy-L-phenylalanyl-L-glutamic acid, carbobenzoxyglycyl-L-glutamic acid, and glutathione were incubated overnight with conjugase, no liberation of glutamic acid could be detected either by the titration method or by manometric means with decarboxylase.

Hydrolysis of glutamic acid was demonstrated with the substrates, *p*-aminobenzoyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic acid, synthetic pteroyltriglutamate, and a natural fermentation factor<sup>5</sup> (Table I).

From the results presented in Table I, it was concluded that conjugase

<sup>2</sup> Obtained through the courtesy of Dr. I. C. Gunsalus.

<sup>3</sup> One sample was obtained through the courtesy of Dr. O. Schales. During the early spring the squashes available in Milwaukee were inactive. We had no difficulty in reproducing Dr. Schales' preparation using fresh squash later in the season.

<sup>4</sup> Obtained through the courtesy of Dr. J. S. Fruton.

<sup>5</sup> All three substrates were obtained through the courtesy of Dr. E. L. R. Stokstad.

hydrolyzes the terminal glutamic acid, having both carboxyl groups free and linked through its amino group to the  $\gamma$ -carboxyl group of the preceding glutamic acid

TABLE I  
*Substrates Attacked by Conjugase*

Substrate	Time	Glutamic acid recovered	-COOH groups liberated	<i>Streptococcus faecalis</i> R <sub>1</sub> active material
	hrs	equivalent	equivalent	equivalent
1 <i>p</i> -Aminobenzoyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic acid	19	0 75		
2 " "	1	0 45		
	2	0 71		
	4	0 71		
	6	0 75		
3 " "	4	0 40	0 53	
	19	0 49	0 63	
	42		1 00	
Synthetic pteroyltriglutamate	19	0 68		0 60
Natural fermentation factor	19	0 50		0 65

TABLE II

*Effect of Increased Amounts of Conjugase on Liberation of Glutamic Acid from p-Aminobenzoyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic Acid*

All the values are in microliters of carbon dioxide liberated and are corrected for a blank containing the same amount of substrate as the experimental tube and conjugase inactivated by heat

	Experiment 1	Experiment 2	Experiment 3
1 5 micromoles triglutamate, 100 $\gamma$ conjugase, incubated 2 hrs, followed by 3 additions of 100 $\gamma$ conjugase every 2 hrs	103	112	79
2 Substrate and conjugase as in No 1, incubated 2 hrs, followed by 3 additions of 0 1 cc 0 01 M calcium chloride	94	111	61
3 Substrate and conjugase as in No 1, no further additions of either conjugase or 0 01 M calcium chloride	76		58

It is of interest to point out that with substrates producing the microbologically active substance the relationship of the liberated folic acid and glutamic acid was closer to 1 1 than 1 2 Findings of Hutchings *et al* (5) that on an equimolecular basis pteroyldiglutamate is just as active micro-

biologically as the monoglutamate explain our results. It seems quite possible that under the ordinary conditions, in which determinations of folic acid were carried out with the aid of chicken pancreas conjugase, the substance actually determined microbiologically has been the diglutamate rather than the monoglutamate.

In none of the experiments reported in Table I was glutamic acid liberated in amounts greater than 1 equivalent. This could not have been due to an insufficient amount of conjugase (Table II). Further addition of enzyme produced a slight increase in liberated glutamic acid in Experiments 1 and 3, in which the reaction stopped on the level of less than 1 equivalent,

TABLE III

*Inhibitory Effect of Glutamic Acid on Conjugase\**

The results represent the difference between the experimental and control tubes and are expressed in microliters of carbon dioxide liberated.

Experiment No	No glutamic acid added	2.5 micromoles added	5 micromoles added	10 micromoles added
1	82	85	23	
2	80	64		
3	105	124	26	
4	116			23

\* The experiments were carried out as follows: 0.1 cc (5 micromoles) of *p*-amino benzoyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic acid was incubated with 0.1 cc (100  $\gamma$ ) of conjugase in borate buffer, pH 7.8, made 0.02 M with respect to calcium chloride, for a period of 20 hours. Variable amounts of glutamic acid (as the sodium salt) were added to the conjugase solution. For each experimental tube, an identical control tube was set up, containing conjugase inactivated by heat.

but was without effect in Experiment 2, where the reaction reached 1.0 equivalent.

Two possible alternatives were therefore considered: (1) the conjugase was unable to hydrolyze the second glutamic acid since it required at least three glutamic acids in a peptide chain, or (2) the reaction did not go to completion because it was inhibited by the glutamic acid formed.

The second alternative seemed more likely. The addition of glutamic acid to the reaction mixture (Table III) showed an inhibitory effect beginning at 5 micromoles of glutamic acid. This amount corresponded to the liberation of 1 equivalent of glutamic acid from the peptide. At present, however, no definite statement can be made as to whether the inhibitory effect of glutamic acid is due to the existence of a true equilibrium for this reaction.

## SUMMARY

Preparations of partially purified conjugase did not liberate the terminal glutamic acid linked to the  $\alpha$ -carboxyl group of the preceding amino acid from any of the four peptides investigated. No hydrolysis of the initial glutamic acid linked either through the  $\alpha$ -carboxyl, *e g* glutamylglutamic acid, or through the  $\gamma$ -carboxyl, *e g* glutathione, was observed.

Conjugase hydrolyzed glutamic acid from *p*-aminobenzoyl- $\gamma$ -glutamyl- $\gamma$ -glutamylglutamic acid, synthetic pteroyltriglutamate, and natural fermentation factor, but more than 1 equivalent of glutamic acid was never recovered. This was probably due to the inhibition of conjugase by the glutamic acid formed during the course of the reaction.

From the evidence thus far accumulated, chicken pancreas conjugase should be classified as  $\gamma$ -glutamic acid carboxypeptidase, requiring at least 2 terminal glutamic acid molecules in the peptide chain. Further requirements for specificity of conjugase with respect to the length of the chain were discussed.

The authors wish to acknowledge the help given by Miss J M Lemley in starting the microbiological assays in this laboratory.

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# BIOCHEMICAL STUDIES ON REGENERATING LIVER

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Regenerating liver of the rat has long been recognized as a source of relatively abundant quantities of homogeneous rapidly growing tissue. Following the removal of the two largest lobes of the liver, the remaining ones, constituting about one-third of the total, enlarge to the size of the original liver in the span of a few days. The work of Higgins and Anderson (1), Brues *et al* (2), and others has established that this enlargement is due to true hyperplastic growth.

It therefore seemed to us an especially favorable tissue in which to test, by chemical analysis, the current view that nucleic acids are intimately related to growth. It should be noted that, although there is considerable indirect evidence to support this view, there is surprisingly little direct chemical data to demonstrate this relation. Caspersson and his colleagues (3-5) have shown an increased absorption of ultraviolet light, at 260 m $\mu$ , in the cytoplasm of actively growing cells. Stowell (6), using photometric histochemical methods, demonstrated a higher nucleic acid content in neoplastic tissue than in the homologous normal tissue. Brachet (7) has combined cytochemical techniques with the chemical estimation of furfural to reveal an increasing pentosenucleic acid (PNA) concentration during embryonic growth. Davidson and Waymouth (8) have estimated the PNA and desoxypentosenucleic acid (DNA) content of embryonic and adult tissues, and have found the concentration of both nucleic acids higher in embryonic than in adult tissues. Schneider and Klug (9) assayed rapidly growing tumors chemically and found them to have a high nucleic acid content.

In this paper we shall present results which demonstrate a rise in PNA content of the liver when it is rapidly growing. We shall also present analyses, performed on the same tissue, for other substances which may be related to growth, lactic acid, glycogen, adenosine triphosphate, adenosine diphosphate, adenylic acid, and pentose phosphate, and for four oxidative enzyme systems, succinoxidase, malic dehydrogenase, cytochrome reductase, and oxalacetic oxidase.

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## EXPERIMENTAL

The Sprague-Dawley albino rats used in these experiments ranged in weight from 108 to 205 gm. Partial hepatectomy was performed by the method of Higgins and Anderson (1). The animals were kept on their customary dog chow and mixed grain diet before and after surgery.

In the first series of experiments, the liver lobes removed at partial hepatectomy were frozen in liquid air, weighed, and stored at  $-20^{\circ}$ . The animals from which they were removed were kept for periods ranging from 13 hours to 23 days. They were then anesthetized lightly with nembutal. Their livers were removed, frozen at once in liquid air, and stored at  $-20^{\circ}$ . Both the animal and the liver removed were weighed at the time of partial hepatectomy and again when the animal was killed. Both the original and regenerated livers of the thirty-eight animals in this series were analyzed.

The frozen tissue was powdered, homogenized, and extracted with cold trichloroacetic acid by the method of LePage and Umbreit (10). An aliquot of the powder was used to determine the water content of the samples. The acid extract was discarded and the tissue residue analyzed for PNA and DNA by the method of Schneider (9, a).

The analyses for nucleotides, pentose phosphate, lactic acid, and glycogen were made by the method of LePage and Umbreit (10) on selected samples of regenerated livers. They could not be done profitably on the original livers, since the ether anesthesia necessary for the surgical procedure is likely to alter the adenosine triphosphate-adenylic acid balance and the lactic acid levels of the cells.<sup>1</sup> For controls, therefore, the livers were removed from two animals in the same fashion as the regenerated livers. The livers were pooled, frozen, and analyzed in the same way as the regenerated livers.

In the second series of experiments, eight animals were used. Four were kept for 48 hours after partial hepatectomy, two for 24 hours, and two for 15 hours. The original livers and regenerated livers were tested for enzyme activity within a short time after removal from the animals. The enzyme activities were determined manometrically by the method of Schneider and Potter (11) for succinoxidase, of Potter (12) for malic dehydrogenase and cytochrome reductase,<sup>2</sup> and of Potter<sup>3</sup> for oxalacetic oxidase.

*Results*

*Pentose Nucleic Acid*.—Fig. 1 indicates the quantity of PNA, expressed in mg per gm of dry tissue, for the original livers and regenerated livers,

<sup>1</sup> LePage, G. A., unpublished.

<sup>2</sup> We wish to thank Mr. Morris Rhian for the purified malic dehydrogenase preparation and Dr. G. A. LePage for the diphosphopyridine nucleotide which were used in these assays.

<sup>3</sup> Potter, V. R., to be published.

plotted against days after partial hepatectomy. It can be seen that during the 1st day and after the 4th day there is no segregation of solid and open circles. But between the 1st and 4th days they are clearly separated, with the concentration of PNA in the regenerated liver considerably higher than that of the original livers. The highest concentration was 44 mg per gm, or 44 per cent of the dry weight of the tissue.

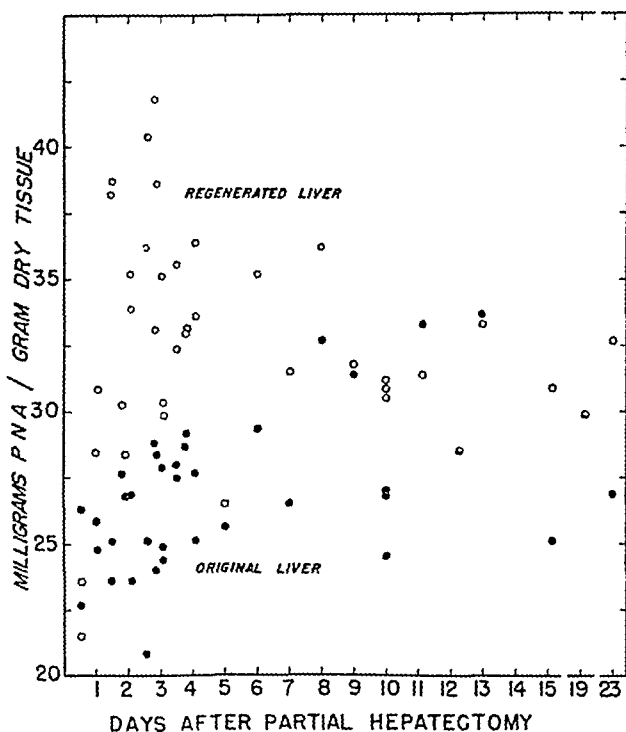


Fig 1 Pentosenucleic acid (PNA) concentration of original (●) and regenerated (○) livers, from  $\frac{1}{2}$  day to 23 days after partial hepatectomy

For each animal, the percentage change in PNA concentration in the regenerated liver as compared with the original liver was calculated. The lower curve of Fig 2 shows the data obtained, plotted against time. It is evident that the rate of increase is greatest between  $1\frac{1}{2}$  and 3 days after partial hepatectomy. The curve has leveled off almost completely between 4 and 5 days. The greatest percentage increase was 62 per cent, obtained in two animals, one 36 hours and the other 62 hours after partial hepatectomy.

Fig 2 also shows the percentage change in the weight of regenerated liver when compared with the weight of the liver left in the animal at partial hepatectomy. The relation, total liver weight =  $1.46 \times$  weight of lobes

removed at partial hepatectomy, seems fairly well established (2, 13), and was the basis for our calculations. Changes in liver weight were corrected for changes in total body weight. It is clear that liver growth, as measured by the organ's weight, is most rapid between  $1\frac{1}{2}$  and 3 days.

Thus, there is a correlation between the time of most rapid growth of the liver and the time of increase in the concentration of PNA.

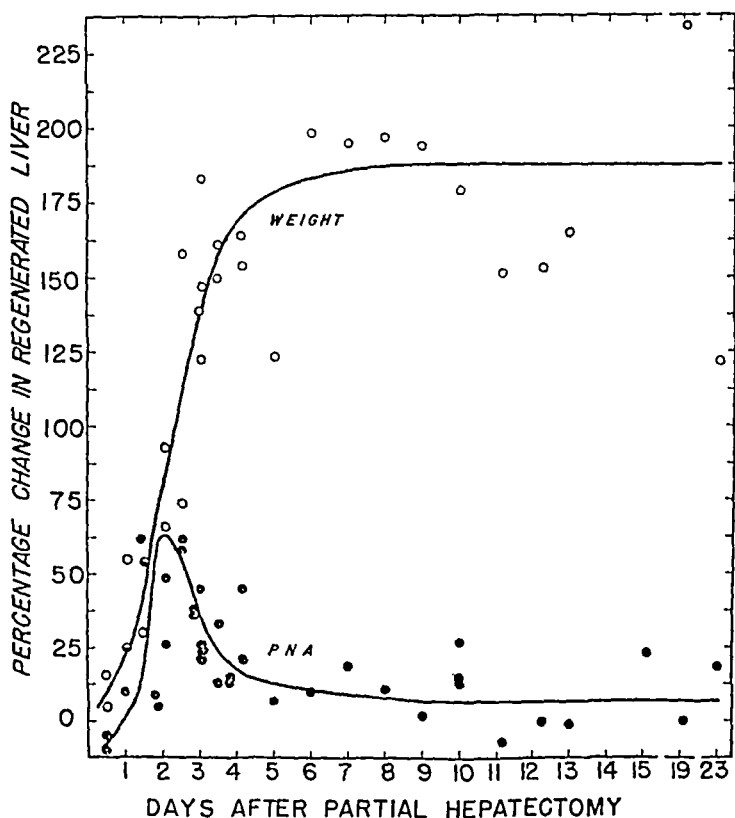


FIG 2 Percentage change in PNA concentration and in weight, in the regenerated liver as compared with the original liver

*Desoxyribonucleic Acid*—Although there were decided changes in DNA concentration following partial hepatectomy, no correlation can be made between the rate of regeneration and the DNA concentration. From Table I it can be seen that both increases and decreases were observed, during, before, and after the  $1\frac{1}{2}$  to  $3\frac{1}{2}$  day period when growth was rapid.

*Nucleotides, Pentose Phosphate, Lactic Acid, and Glycogen Analyses*—The samples of regenerated livers were chosen for these analyses so as to include animals in which growth had not yet begun, those in which it was occurring rapidly, and those in which it was essentially complete. In all thirteen

animals were studied, three between  $\frac{1}{2}$  to 1 day after partial hepatectomy, before growth had begun and with no significant increase in PNA concentration, seven animals between  $1\frac{1}{2}$  and 4 days when growth was rapid and with increases in PNA concentration ranging from 26 to 62 per cent,

TABLE I  
*Changes in Desoxyribonucleic Acid in Regenerated Liver*

Days after partial hepatectomy	No. of animals	Per cent change in DNA
$\frac{1}{2}$ - 1	4	-10, -15, -4, -2
$1\frac{1}{2}$ - $3\frac{1}{2}$	18	+37, +30, -21, -24, +2, -2, +15, +7, -16, +4, +108, +27, +2, +12, +23, +28, +10, +10
4-23	16	+15, -30, +29, -4, +10, -2, +21, +45, +9, +7, +29, -14, +6, +19, -3, +34

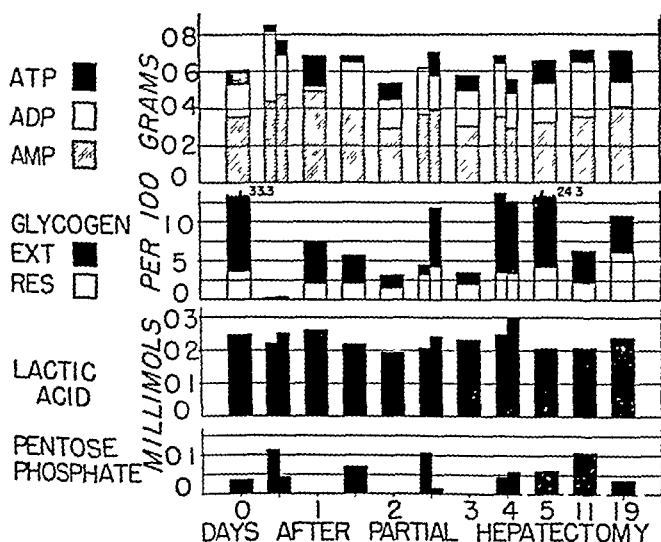


FIG 3 Analyses of selected samples of regenerated liver for adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenylate acid (AMP), glycogen, lactic acid, and free pentose phosphate

and three animals between 5 and 19 days, after growth had slowed and with no significant increase in PNA concentration

Fig 3 gives the results of these analyses. All quantities are indicated in micromoles per 100 gm of wet tissue. The first column shows the analyses of the pooled control livers. Narrower columns are used when two animals of the same postoperative age were analyzed.

The most significant features of these data are (1) the uniform lactic acid levels, whether the livers were rapidly growing or not, from 200 to 300 micromoles per cent, and (2) the absence of any significant differences between the rapidly growing and relatively non-growing livers in the total nucleotide concentration as well as the balance among adenosine triphosphate, adenosine diphosphate, and adenylic acid

The variation in concentration of free pentose phosphate is similar to what one finds among control animals (14). The same is true of glycogen, with the exception of the animals killed a half day after partial hepatectomy. These livers contained very little glycogen, none at all of the extractable form and almost none of the non-extractable form.

*Enzyme Systems*—To ascertain whether the frozen livers, samples of which had been used for the chemical analyses, could be profitably used for

TABLE II  
*Effect of Freezing on Enzyme Activities*

	$Q_{O_2}$ , fresh liver	$Q_{O_2}$ , frozen liver	Decrease
			per cent
Succinoxidase	75.3	37.8	50
Malic dehydrogenase	103.6	86.0	17
Cytochrome reductase	126.3	82.4	35
	mg per 100 gm	mg per 100 gm	
Pentosenucleic acid	824	825	
Desoxypentosenucleic acid	245	247	

these enzyme studies, an experiment was performed on the effect of freezing the liver in liquid air and thawing it, before preparing the homogenate. The results are given in Table II. The nucleic acid assay of the homogenates prepared from the fresh and frozen liver is included, for it serves as a check on the equality of tissue concentration in the two homogenates. The succinoxidase activity is reduced by 50 per cent, and the cytochrome reductase by 35 per cent. The reduction noted in malic dehydrogenase activity may be simply the result of lowering the cytochrome reductase activity to the point where it no longer is present in excess, as it needs to be when assaying for malic dehydrogenase (12). This is indicated by the essentially equal  $Q_{O_2}$  values for the activities of both enzyme systems. Similarly, in all but one of the nine pairs of frozen original and regenerated livers assayed, the activities of both enzymes were reduced and equal. For some reason, the cellular structure of this one liver resisted alteration by freezing and thawing, with the result that the cytochrome reductase activity was not reduced. Here the malic dehydrogenase showed its full normal activity.

TABLE III  
Enzyme Activity of Regenerated Liver

	Time of regeneration							
	15 hrs	15 hrs	23 hrs	23 hrs	48 hrs	48 hrs	48 hrs	48 hrs
H O content, %	(a) 71.2 (b) 68.8	72.5 74.8	70.9 71.7	72.6 73.5	71.5 74.3	73.0 75.8	72.9 72.0	71.9 72.5
PNA, mg per gm dry tissue	(a) 2.96 (b) 2.37	2.87 2.77	2.53 3.14	3.03 4.30	2.89 4.33	3.58 4.40	3.63 3.89	3.49 4.02
Change from original, %	-20	-4	+24	+42	+50	+19	+7	+13
DNA, mg per gm dry tissue	(a) 1.01 (b) 0.75	1.10 0.97	0.79 0.73	1.00 0.87	0.83 0.74	1.07 0.84	1.13 0.77	1.17 0.90
Change from original, %	-26	-12	-8	-13	-11	-22	-32	-23
Q <sub>o</sub> , succinoxidase	(a) 76.1 (b) 52.3	71.8 49.8	83.8 64.2	81.0 64.5	78.4 66.4	86.0 76.6	64.7 61.1	75.5 70.8
Change from original, %	-31	-31	-23	-20	-15	-11	-6	-6
Q <sub>o</sub> malic dehydrogenase	(a) 103.4 (b) 63.6	103.6 72.4	85.6 65.0	97.0 76.9	97.7 72.5	95.1 68.2	79.7 50.5	84.7 58.1
Change from original, %	-39	-30	-24	-21	-26	-28	-37	-31
Q <sub>o</sub> , cytochrome reductase	(a) 132.5 (b) 76.1	137.1 85.0	112.4 80.9	111.3 81.5	97.2 75.3	102.1 80.3	97.6 53.1	95.4 61.4
Change from original, %	-43	-38	-28	-27	-23	-21	-46	-36
Q <sub>o</sub> , oxalacetic oxidase	(a) (b) 10.9	 18.7	12.5 11.8	15.0 10.9	18.6 17.0	20.4 17.6	18.5 15.1	19.9 18.8
Change from original, %			-6	-27	-9	-14	-18	-6

(a) original, (b) regenerated

Potter<sup>3</sup> has observed that freezing reduces the activity of the oxalacetic oxidase system effectively to zero

No differences were found between the enzyme activities of the original



and regenerated livers in the series of frozen livers, except for the succinoxidase activities in two animals. In both, the animals had been sacrificed before growth of the liver had begun and before the PNA concentration had increased. One showed a fall in succinoxidase  $Q_{O_2}$  from 22.4 in the original liver to 3.4 in the 13 hour regenerated liver. In the other, the 24 hour regenerated liver showed a fall in succinoxidase  $Q_{O_2}$  from 28.1 to 6.6. Whether any significance was to be attached to this decrease could be determined only by assays of fresh tissue.

In the second series of experiments, the livers, original and regenerated, were dropped into ice-cold isotonic  $KCl^4$  as soon as they were removed from the animals, and shortly thereafter homogenized. Two homogenates of each liver were prepared, one with isotonic  $KCl^4$  for the oxalacetic oxidase assay, the other with distilled water for the assays of the other three enzymes. Samples of the livers were also taken for determination of water content.

Table III gives the results, together with those of the nucleic acid determinations.

In these animals, liver regeneration, as estimated by the weight of the regenerated organ, was more rapid than in the animals of the first series. This may account for the somewhat larger PNA increases observed in the 24 hour regenerated livers. It may also explain the low PNA increases found in two of the four 48 hour regenerated livers, in these two animals, the rate of regeneration may already have fallen off. In these animals, too, the concentrations of nucleic acid in the original livers were higher than most of those in the first series of animals.

The activities of all four enzyme systems were lower in the regenerated liver than in the original livers. This was true even in the 15 hour livers which did not yet show PNA increases. There was some indication that the activities may be lower at this time than later, when regeneration is very rapid. This is also suggested by the low succinoxidase activities of the two frozen regenerated livers which we described earlier. The indication of a return to normal values for succinoxidase  $Q_{O_2}$ , seen by the values in the 48 hour regenerated livers, is supported by unpublished observations made earlier by one of us (V. R. P.) in which reductions in succinoxidase  $Q_{O_2}$  at 21 hours were followed by a return to normal values at 3 and 4 days.

#### DISCUSSION

Our study differs from those of other investigators in that we have compared the regenerated liver with the control liver of the same animal. This procedure provides us with only two points on the time curve for the process.

<sup>4</sup> The  $KCl$  was made slightly alkaline (pH 7.7 to 8.1) by the addition of 8 cc. of 0.01  $M$   $KHCO_3$  per liter.

of liver restoration in the individual, and it is only by examining the data as a whole that the correlation between the period of rapid growth and the increase in PNA concentration is apparent. Furthermore the composite data indicate a lag phase in the growth as well as in the PNA increase. Thus the individual variations encountered may well be the result of a variable lag phase in the individual rats, with the maximum rate of regeneration occurring at different points on the time curve. Thus by determining the per cent increase in PNA by analyzing both the original and regenerated liver we may have an index of the rate of growth for the individual at the time the animal was killed.

On the other hand, the data indicate that the rapid growth of liver, following partial hepatectomy, does not involve an appreciable change in the levels of lactic acid, adenosine triphosphate, adenosine diphosphate, adenylic acid, and free pentose phosphate. However, important biochemical changes take place in the liver cells before active growth begins and before the PNA content rises. The activities of all four of the oxidative enzyme systems we have studied drop markedly, at least when measured *in vitro*. It is at present not possible to say whether these enzymatic changes are related to the morphological changes which occur in the liver cells at this time, such as the deposition of fat droplets in the cytoplasm.<sup>5</sup>

In contrast to the findings with PNA, the DNA changes were not correlated with the rate of growth, nor have we attempted to correlate the differences in different animals with the histological and cytological picture of the particular liver samples.

Our data, while establishing that the PNA concentration does increase during growth of the liver, fail to throw much light on the mechanism linking the two. The increase in PNA concentration can account, at least in part, for the marked increase in  $P^{32}$  uptake which Brues *et al* (15, 16) observed in the ribonucleic acid fraction of regenerating liver. It can also explain the increased ultraviolet absorption at 260  $m\mu$ , observed by Stowell (17) in the cytoplasm of regenerating liver cells. Our observations that the nucleotide concentration does not rise during regeneration indicates that this increased absorption is, in fact, due to nucleic acid. Finally, the increased PNA content during liver regeneration accounts for the increased staining with pyronin noted by Brachet (18).

Davidson and Waymouth (19) reported that the PNA was not significantly increased in samples of regenerating liver, while the acid-soluble nucleotides showed increases. Whether the differences between their data and ours are due to differences in analytic method (see Schneider (20)) or

<sup>5</sup> Large numbers of fat droplets are visible in homogenates of livers of this post-operative age.

to the fact that we used samples of the original liver for controls cannot be decided at this time

#### SUMMARY

1 During the time of most rapid growth of regenerating liver, the concentration of pentosenucleic acid increases

2 The desoxypentosenucleic acid content does not change in a consistent fashion during liver regeneration

3 No significant changes occur during liver regeneration in the content of lactic acid, adenosine triphosphate, adenosine diphosphate, adenylic acid, and free pentose phosphate

4 Following partial hepatectomy there is a marked decrease in activities of succinioxidase, malic dehydrogenase, cytochrome reductase, and ovalaccic oxidase systems, as tested *in vitro*

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# CHANGES IN NUCLEIC ACID CONCENTRATION DURING THE DEVELOPMENT OF THE CHICK EMBRYO

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Many investigators have been concerned with the changes in nucleic acid concentration which occur during embryonic development (see Needham (1) for a review of the older work and Needham (2) and Brachet (3) for reviews of the more recent literature). However, they have had to rely upon indirect and largely inadequate means of estimating the concentrations of these substances, such as the pentose content, purine nitrogen relative to total nitrogen, individual purine and pyrimidine bases, or residual phosphorus following acid extraction. Recently, more reliable methods of analyses for the nucleic acids have become available (4, 5).

In this paper we are presenting quantitative data for pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA), from the 2nd through the 20th day of development. They will show that the concentration of these acids is highest at about the 15th day, rising to this peak during the period of most active protein metabolism in the embryo. They will also show that during the period when the embryo is said to be richest in growth-promoting substances it has the highest levels of nucleic acids.

## EXPERIMENTAL

Eggs of pure bred single comb white Leghorn hens were incubated under constant conditions of temperature ( $37-38^{\circ}$ ) and humidity for lengths of time varying from 32 hours to 20 days<sup>1</sup>. The embryos were removed from the yolk, dropped into ice-cold physiological saline, dissected free from all extraembryonic tissue, touched to damp filter paper to remove most of the saline, and dropped into liquid air. All these manipulations were made rapidly, in a refrigerated room. The embryos were stored at  $-20^{\circ}$  until analyzed.

The frozen embryos were powdered in a steel cylinder, chilled with liquid air, and then loosely homogenized in trichloroacetic acid (6). They

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<sup>1</sup> We are indebted to Professor W. W. Cravens and the Poultry Department, University of Wisconsin, for generously supplying and incubating the many eggs which these experiments required.

were analyzed for PNA and DNA by the method of Schneider (4) The quantities of trichloroacetic acid used in the extractions of acid-soluble constituents and of nucleic acids were adjusted in order to keep the proportion of acid to tissue approximately the same in all cases

Fig 1 shows the concentrations of PNA and DNA, in micrograms per embryo, plotted against the days of incubation of the egg The portion of

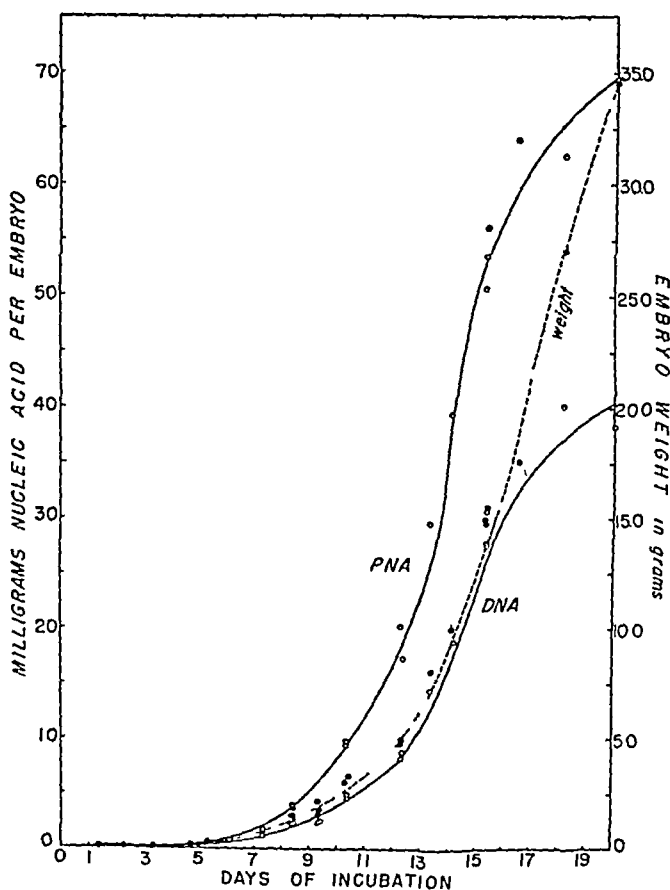


FIG 1 Changes in the pentosenucleic acid (PNA) concentration, deoxypentose-nucleic acid (DNA) concentration, and weight of the chick embryo from the 2nd to the 20th day of incubation

the curve for the first 9 days is enlarged in Fig 2 The PNA concentration per embryo increases some 16,000 times and DNA some 30,000 from the end of the 1st day (32 hours) to the 20th day of incubation Not much significance can, however, be attached to these increases for, as can be seen from the figures, the weight of the embryo has increased in roughly the same manner

Fig 3 shows the concentration of PNA and DNA, in mg per 100 gm of

dry tissue, plotted against days of incubation. The DNA concentration roughly doubles between the 2nd and 15th days, from approximately 100 to 200 mg per cent, and then falls sharply, so that it is back to the original value by the 20th day. The PNA data for the first few days suggest a fall from about 350 mg per cent on the 2nd day to less than 200 mg per cent on the 5th day. From the 5th to the 14th day, the PNA concentration

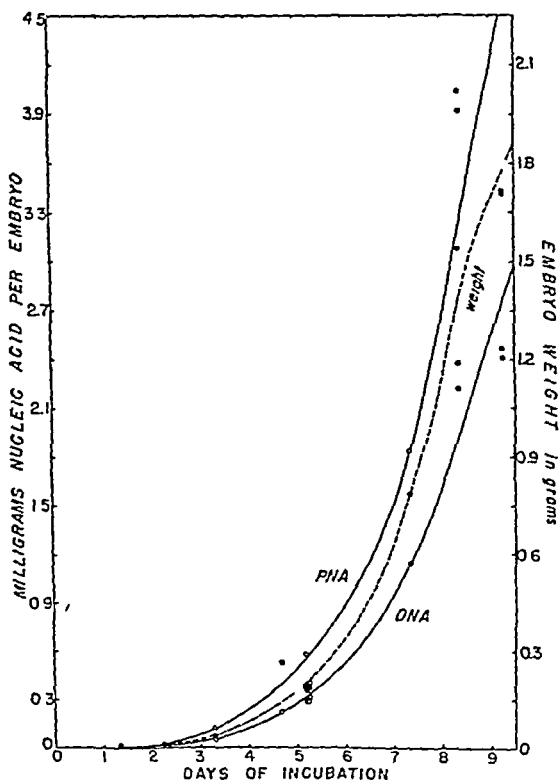


FIG 2 Portion of Fig 1 extending from the 2nd to the 9th day of incubation, enlarged

rises sharply, so that it approximately doubles in that time, from 200 to 400 mg per cent. It then declines sharply and is back to 200 mg per cent by the 20th day.

We performed a number of experiments to determine whether the weight of the inert feathers could account for the fall in nucleic acid content, per unit of embryo weight, after the 14th to the 15th day. We removed the skin and feathers from 12 and 14 day embryos, and the feathers alone from 16 day embryos. The values obtained for PNA and DNA were not appre-

ciably different from those obtained with feathered embryos. Furthermore, the weight of the feathers, even on the 19th day, is not large enough, in relation to total embryo weight, to lower the DNA and PNA values very much. We made no attempt to evaluate the influence of other inert structures, such as bones, which are enlarging rapidly at this time.

Such analyses of the whole organism must obviously obscure important changes which occur in individual organs. Yet it is probably significant that the period when both PNA and DNA concentrations are rising is the

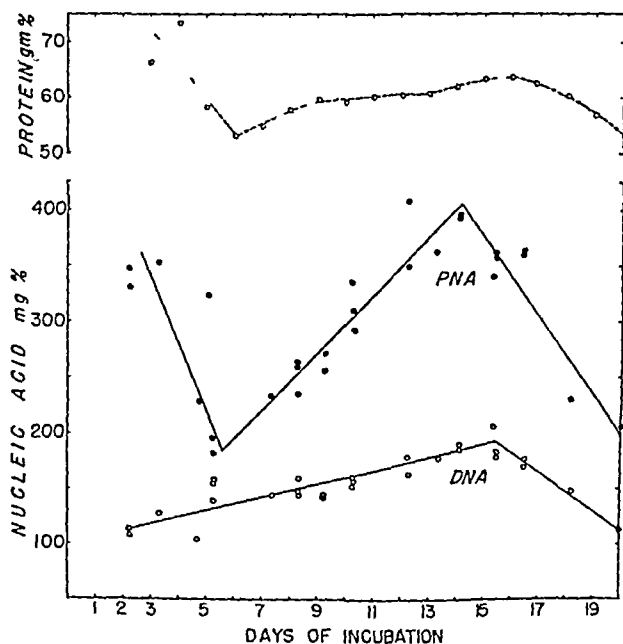


FIG 3 Changes in pentosenucleic acid (PNA) and desoxypentosenucleic acid (DNA) concentrations during the development of the chick embryo. Top curve, changes in protein concentration, from data of Needham (1). Dry weight basis.

period when protein metabolism plays its important part in the development of the embryo.

Needham (1) has assembled a great deal of data to indicate an ontogenetic succession of carbohydrate, protein, and fat as the chief sources of material both for embryonic architecture and for catabolic fuel. In Fig 3, top curve, we have plotted the data Needham gives for the protein content of the embryo, in mg per cent dry weight. Its general similarity to the PNA curve is striking.<sup>2</sup> When the data for carbohydrates and fats are similarly

<sup>2</sup> In plotting this same data, Needham subtracted the feather proteins so that the protein content of the embryo dropped off sharply after the 11th day. However, if we consider the curve to indicate protein synthesis by the embryo, there seems to be no reason for excluding the feather proteins.

plotted, they show no resemblance whatever to the nucleic acid changes, the carbohydrate content is high at the start and falls to a low level by the 8th day and the fat increases slowly from the start and more quickly after the 15th day

Although fat is the chief energy source for the chick embryo, Needham (1) showed that carbohydrate and protein were the energy sources of the early stages of development. Determination of the nitrogenous waste products showed a sharp rise in the protein combustion by the embryo, per unit weight, per day, from the 6th to the 8th or 9th day, and then a gradual fall to the 15th day. Thus, the 6th to the 15th day would appear to be the time when protein catabolism occurs in the embryo. Needham points out that a given weight of embryo catabolizes six or seven times as much protein on the 8th day of development as it does on the 4th or the 16th day.

The finding that the content of nucleic acid, especially PNA, rises only during the period of embryonic development when protein metabolism is most conspicuous is in harmony with the current view that PNA is related to protein synthesis. But it gives no information concerning the causal relation between the two.

Needham (1, 2) has discussed the work demonstrating that certain periods of development are better than others for extracting from the chick embryo the growth-promoting substances stimulating the growth of cells in tissue culture. In view of suggestions that this growth-promoting capacity of chick embryo extract may be associated with pentosenucleoproteins (7-9), it is interesting that the period of maximal growth-promoting power of the embryo cells lies between the 11th and 17th days. This is the period when the PNA concentration is highest, between 300 and 400 mg per cent.

#### SUMMARY

- 1 The pentosenucleic acid and desoxypentosenucleic acid contents of chick embryos have been determined, from the 2nd to the 20th day of development.
- 2 The DNA concentration, when expressed in mg per cent of wet tissue, rises from the 2nd to the 15th day, and then falls sharply. The PNA falls from a high level on the 2nd day to a low one on the 5th, it then rises to a maximum on the 14th day and falls off sharply.
- 3 The relations of these changes in nucleic acid concentration to (1) the period of protein metabolism in the embryo and (2) the optimum period for extracting growth-promoting substances are discussed.

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# PHOSPHORYLATING GLYCOLYSIS IN THE EARLY CHICK EMBRYO\*

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From an extensive series of experiments with whole embryos and with tissue mince, Needham and his coworkers (1-7) concluded that in the early chick embryo the phosphorylation route of glycolysis, the Meyerhof-Embden cycle, is imperfectly established. Because glycogen generally failed to be converted to lactic acid, they argued that the enzyme esterifying glycogen was absent or insufficient. Since they could demonstrate no breakdown of hexose diphosphate beyond triose phosphate, they maintained that the "dismutase" forming phosphoglycerate and lactate from triose phosphate and pyruvate was similarly lacking or insufficient. Though extracts of chick embryo were strongly positive with the glucose dehydrogenase and growth rate tests with *Bacillus influenzae*, they gave negative results with the malic dehydrogenase system. The authors therefore concluded that coenzyme I, essential for the operation of the Meyerhof-Embden cycle, was absent from the chick embryo. Their study of the phosphorus compounds in trichloroacetic acid extracts of the embryo revealed between 5 and 7 mg. per cent of adenylyl pyrophosphate phosphorus. This quantity the authors presumably considered insufficient for the phosphorylation pathway in glycolysis, since they listed as the fourth deficiency in this pathway the lack of adenylyl pyrophosphate.

In 1940, Meyerhof and Perdigon (8) succeeded in preparing extracts of early chick embryos which would readily glycolyze hexose diphosphate, in the presence of added coenzyme I. The ability of these extracts to form lactic acid from hexose diphosphate and pyruvate demonstrates the presence of the triose phosphate dehydrogenase which Needham considered lacking. In view of Needham's emphasis on the importance of cell structure for the presumed non-phosphorylating glycolysis mechanism but not for the phosphorylating one, it is interesting to note how Meyerhof and Perdigon extracted the embryos. To obtain actively glycolyzing extracts, they had

\* This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

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to freeze the embryos twice, the first time before grinding with sand, and again after adding water or saline to the ground tissue

Meyerhof and Peidigon also succeeded in demonstrating the presence of coenzyme I, using the fermentation rate of dried yeast as the method of assay. Comparing the  $\text{CO}_2$  production by the yeast apoenzyme after the addition of boiled water extract of embryo with that produced when known quantities of coenzyme I were added, they calculated that the embryo, from the 4th to the 9th day of development, contained about 1.5 to 3 mg per gm. of dry tissue.

In this paper, we are presenting data which strengthen the view that phosphorylating glycolysis does occur in the early chick embryo. The data come from two sources: (1) chemical analyses of embryos for the known intermediates in the Meyerhof-Embden cycle and for related compounds, and (2) the behavior of homogenates of chick embryo, fresh and frozen, towards glucose, hexose diphosphate, glucose-6-phosphate, and fructose-6-phosphate, in properly fortified glycolytic systems.

#### EXPERIMENTAL

The eggs used in these experiments came from pure bred flocks of single comb white Leghorn hens, kept at the University of Wisconsin poultry farms.<sup>1</sup> They were incubated under constant conditions of temperature ( $37-38^\circ$ ) and humidity.

*Chemical Analyses*—The embryos were removed from the yolks, dropped into ice-cold physiological saline, dissected free from all extraembryonic tissue, touched to damp filter paper to remove most of the saline, and dropped into liquid air. All these manipulations were made rapidly (in a minute or less) in a refrigerated room. The frozen embryos were stored at  $-20^\circ$  until analyzed. The number of embryos used was varied so that 1.5 to 2.0 gm. were available for analysis. Even a single 10 day embryo was too large for analysis, so that it was divided into two aliquots and each separately analyzed.

The methods of LePage and Umbreit (9) were used to powder the frozen embryos, extract them with trichloroacetic acid, and separate the barium-insoluble fraction and barium-soluble, alcohol-precipitable fractions. Aliquots of the original trichloroacetic acid extract were taken for the determination of "true" inorganic phosphorus, phosphopyruvic acid, total phosphorus, and lactic acid. Aliquots of the barium-insoluble fraction were used to measure fructose, inorganic phosphorus, easily hydrolyzable phosphorus (7 minutes in 1 N HCl at  $100^\circ$ ), phosphorus resistant to acid

<sup>1</sup> We are indebted to Professor W. W. Cravens and the Poultry Department for generously supplying and incubating the many eggs which these experiments consumed.

hydrolysis (3 hours in 1 N HCl at 100°), pentose, and adenosine. Aliquots of the barium-soluble, alcohol-precipitable fraction were used to determine fructose, coenzyme, reducing sugar, reducing sugar after 7 minute hydrolysis in 1 N HCl, phosphocreatine, "true" inorganic phosphorus, easily hydrolyzable phosphorus, alkali-labile phosphorus (20 minutes in 1 N KOH at room temperature), phosphopyruvic acid, total phosphorus, pentose, and adenosine. The tissue residue was analyzed either for glycogen or for nucleic acids (10). The nucleic acid data are included in another publication (11). All manipulations up to the time aliquots were taken were made either in the refrigerated room or in containers surrounded by ice. The determinations of "true" inorganic phosphorus, easily hydrolyzable phosphorus, alkali-labile phosphorus, phosphopyruvic acid, and phosphocreatine were made first, as soon as the fractions containing them were separated.

The methods of determination, with the exceptions to be noted, are those described by LePage and Umbreit (9), scaled down to an even more "micro" level. After they were found to give the same values as the methods described (9), the method of Lowry and Lopez (12) was used for "true" inorganic phosphorus, and the ultraviolet absorption at 260 m $\mu$  (13) was used to determine adenosine. Both barium-insoluble and barium-soluble, alcohol-precipitable fractions showed absorption maxima at 260 m $\mu$ . The absorption at 340 m $\mu$  by the reduced form was used to determine coenzyme (14) in addition to the nicotinic acid assay described (9).

From the data thus obtained, the concentrations of the following compounds in the original embryos were calculated: glycogen, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexose diphosphate, triose phosphates, phosphoglyceric acids, phosphopyruvic acid, lactic acid, adenosine triphosphate, adenosine diphosphate, adenylic acid, phosphocreatine, free pentose phosphate, coenzyme, inorganic phosphorus, and total acid-soluble phosphorus.

Since neither the nicotinic acid nor spectrophotometric assays for coenzyme distinguishes between diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN), concentrated extracts of 7, 8, and 9 day embryos were tested for DPN in the malic dehydrogenase system (Potter (15)). The barium-soluble fraction of about 25 gm of frozen embryos was prepared and then precipitated with 95 per cent ethanol. The material which did not dissolve in water when brought to pH 5 by glacial acetic acid was centrifuged and discarded. The coenzyme was precipitated by adding an excess of silver nitrate to the supernatant, in the presence of 1 volume of ethanol. The silver was removed from the precipitate with hydrogen sulfide and the barium from the filtrate with dilute sulfuric acid. The volume of the extract was brought to 5 to 6 ml and the pH to 7.4.

The activity of the extracts was determined by measuring the oxygen consumption in the conventional Warburg flasks containing 0.027 M sodium phosphate buffer, pH 7.4, 0.01 M nicotinamide, 0.05 M sodium malate, 0.05 M sodium glutamate,  $4 \times 10^{-5}$  M cytochrome *c*, and 10 mg of liver (as 10 per cent homogenate in distilled water). DPN is required for oxygen uptake (15).

*Glycolytic Activity of Homogenates*—The system used to test the glycolytic activity of embryo homogenates was one suggested by the recent work on brain homogenates by Utter *et al.* (16, 17) and Racker and Krimsky (18, 19). It is a system in which we have been able unequivocally to demonstrate the glycolysis of glucose, hexose diphosphate, and hexose monophosphates by tumor homogenates (20).

We employed 85 to 170 mg of tissue (wet weight) per Warburg flask, in a total volume of 3.0 ml. The contents of the flasks in all cases included the following basic mixture, with final concentrations as indicated: 0.0033 M  $\text{MgCl}_2$ , 0.01 M  $\text{NH}_4\text{PO}_4$ , pH 7.6, 0.05 M KCl- $\text{KHCO}_3$ ,<sup>2</sup> 0.0005 M potassium pyruvate, freshly prepared,<sup>3</sup> and distilled water to bring the volume to 3.0 ml. The following, when used, had the indicated final molarity: adenosine triphosphate (ATP), prepared from rabbit muscle (22), 0.00067 M, hexose diphosphate (HDP), prepared by the method of Neuberger *et al.* (23), 0.008 to 0.001 M, glucose-6-phosphate, synthetically prepared (24)<sup>4</sup> or separated from a hexose monophosphate mixture by differential hydrolysis (25), 0.004 M, fructose-6-phosphate, prepared by the method of Neuberger *et al.* (23), 0.004 M, glucose, 0.028 M, DPN, prepared from yeast (14), 0.00033 M, and nicotinamide, 0.04 M. All ingredients were added to the main chamber of the flask, except for DPN, and, in some experiments, ATP, which were placed in the side arm. The pyruvate and  $\text{MgCl}_2$  additions were delayed until just prior to adding the homogenate. Before these three ingredients were added, the flasks were surrounded with cracked ice.

The homogenates were prepared in an all-glass homogenizer (26) from embryos removed as described earlier, and used fresh or after freezing in liquid air. In some experiments, the homogenate of fresh embryos was divided into two portions, one assayed immediately and the other frozen in liquid air and later thawed and assayed. Both water and isotonic KCl homogenates were used.

The flasks were gassed for 13 minutes with either a 95 per cent  $\text{N}_2$ -5 per

<sup>2</sup> The combined  $\text{KHCO}_3$ -KCl solution was prepared by slowly adding an equal volume of 1 N HCl to 1 M  $\text{K}_2\text{CO}_3$ , and then equilibrating with 5 per cent  $\text{CO}_2$ .

<sup>3</sup> We found, as reported by Needham *et al.* (2) and Elliott and Henry (21), that pyruvate eliminated a slight lag in the initial rate of glycolysis.

<sup>4</sup> We are grateful to Dr. H. A. Lardy for a gift of some synthetic glucose 6 phosphate.

cent CO<sub>2</sub> mixture or a 95 per cent O<sub>2</sub>-5 per cent CO<sub>2</sub> mixture The pH of the medium was 7.5 to 7.6 at the end of this period

The CO<sub>2</sub> output was measured in the Warburg apparatus to determine the rate of acid production during glycolysis A few chemical analyses for lactic acid were also performed by the method of Barker and Summerson (27)

TABLE I  
*Analyses of Chick Embryos*

The values are expressed in micromoles per 100 gm

Age, days	3	5½	5½	8	8	10	Mature rat tissues*
No. of embryos analyzed	67	10	10	2	2	1	
Net weight per embryo, gm	0.032	0.198	0.183	1.539	1.486	3.334	
Glycogen (as glucose)				98	61	122	81-28,450
Glucose-1-phosphate	167	156	144	202	199	170	42-175
Glucose 6 phosphate	193	188	190	195	286	307	185-423
Fructose-6-phosphate	12	12	13	8	8	8	17-53
Hexose diphosphate	2	2	2	2	1	4	4-17
Triose phosphates	291	297	291	29	131	86	
Phosphoglyceric acids	87	130	91	244	68	258	98-209
Phosphopyruvic acid	79	76	82	18	17	35	41†
Lactic acid	366	382	416	250	300	280	141-578
Adenosine triphosphate	98	54	76	43	17	72	8-542
"    diphosphate	2	6	1	42	23	67	27-330
Adenylic acid	124	143	134	149	165	96	144-329
Phosphocreatine	48	63	44	59	110	37	116-1630
Pentose phosphate	0	15	3	18	0	6	22-72
Coenzyme (as DPN)	4	4	6	6	4	6	16-35
Acid soluble phosphorus	1725	1930	2010	1900	1780	2290	2390-5070
Inorganic phosphorus	268	284	416	346	348	800	417-748
Organic phosphorus	1457	1646	1594	1554	1432	1490	1895-4322
"    phosphate accounted for, %	90	77	78	74	75	92	65-81

\* From LePage (28)

† Calculated from data for liver in LePage and Umbreit (9)

### Results

*Chemical Analyses*—The results of the chemical analyses are presented in Table I, which also includes the values obtained by LePage (28) for five differentiated rat tissues, brain, muscle, liver, kidney, and heart All values are given in micromoles per 100 gm of wet tissue

All of the concentrated extracts of 7, 8, or 9 day embryos tested gave positive results for DPN activity in the malic dehydrogenase system The

concentration of DPN in the extracts was estimated by comparing the  $Q_{O_2}$  obtained, when a given amount was added, with the  $Q_{O_2}$  obtained with known amounts of DPN. Up to a concentration of about 120  $\gamma$  of DPN, there is a linear relation between  $Q_{O_2}$  and DPN concentration.

Thus, in one experiment, 0.6 ml of extract was used in one flask, 1.2 ml in a second, and 0.6 ml together with 50  $\gamma$  of DPN in another. They gave DPN values, as read from the  $Q_{O_2}$ -concentration curve, of 43, 55, and 44  $\gamma$  per ml, respectively. These values (average of 47  $\gamma$  per ml)

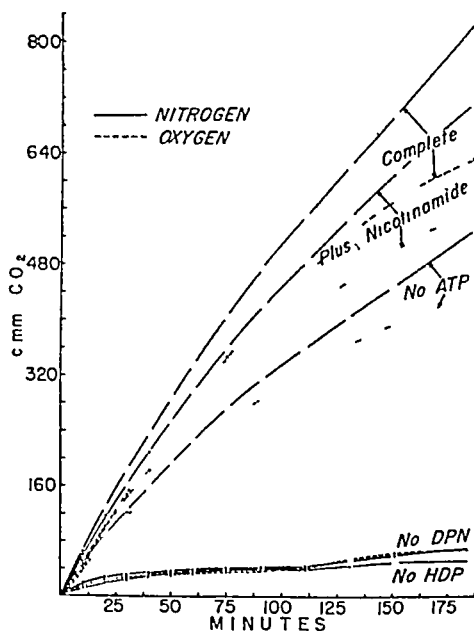


FIG 1 Glycolysis by 8 day chick embryos homogenized in isotonic KCl, frozen, thawed, and tested in nitrogen and in oxygen, with homogenate equivalent to 125 mg wet weight of embryo. The reaction mixture in the "complete" system included glucose, ATP, DPN, and the basic mixture as given in the text, plus HDP at 0.001 M.

were higher than that obtained for the same extract when tested spectrophotometrically (14) for coenzyme, both DPN and TPN, 31  $\gamma$  per ml.

When malate was omitted from the system, no appreciable oxygen consumption could be measured, whether DPN, up to 100  $\gamma$ , or embryo extract was present or absent.

*Glycolytic Activity of Homogenates*—The results obtained with various types of homogenates of pooled chick embryos are shown in Figs 1 to 6. Fig 1 shows the data obtained with KCl homogenates of 8 day embryos that had been frozen and thawed before homogenization. We did not prepare an extract, but used the whole homogenate (*cf* Meyerhof and Perdigon (8)). It was tested in a reaction mixture that included all of the known factors that might be required for glycolysis, and with various omissions,

in oxygen and in nitrogen. The data show that the complete system glycolyzes HDP at a rapid rate that is maintained for 3 hours or more. Nicotinamide is not required to preserve the glycolysis and is actually slightly inhibitory, in marked contrast to the results obtained with tumor (20). Omission of ATP produced a lowered rate of glycolysis but there was still considerable activity. However, omission of DPN or HDP resulted in an almost complete loss of activity. The system thus requires DPN and HDP and, in the absence of HDP, glucose cannot be glycolyzed. However, in the presence of small amounts of HDP, insufficient to maintain the rate of glycolysis alone, glucose can be glycolyzed nearly as well as HDP, pro-

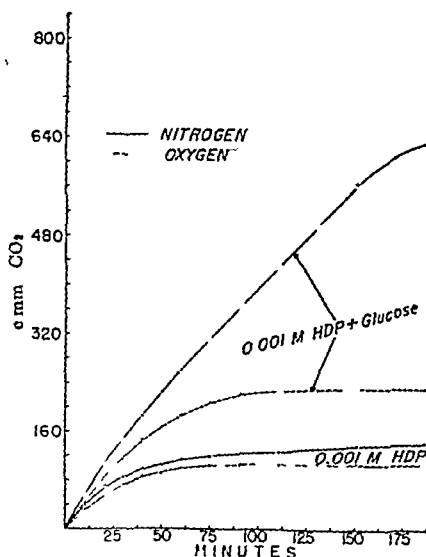


FIG 2 Glycolysis by 8 day chick embryo homogenates in the presence of 0.001 M HDP with and without glucose. Other reactants and homogenate as in Fig 1.

vided the gas phase is nitrogen, in oxygen, the rate was much lower and the  $\text{CO}_2$  output soon ceased altogether (Fig 2). This inhibitory effect of oxygen was present but much less noticeable in the systems that included high levels of HDP (Fig 1), and it is therefore unlikely that the oxygen effect was due to oxygen uptake. Furthermore this possibility was tested by measuring the oxygen uptake of the complete system without bicarbonate, in air, with and without  $4 \times 10^{-5}$  M cytochrome c. The oxygen uptake was very low in each case, and amounted to only 6.3 and 5.3 c mm of  $\text{O}_2$  per 100 mg of embryo in 20 minutes.

Water homogenates are compared with KCl homogenates in Fig 3, in the complete system and with glucose or HDP omitted. In these systems the two types of homogenates gave almost identical results. The



slight inhibition with nicotinamide and the complete loss of activity when HDP is omitted are here shown with water homogenates, and are compa

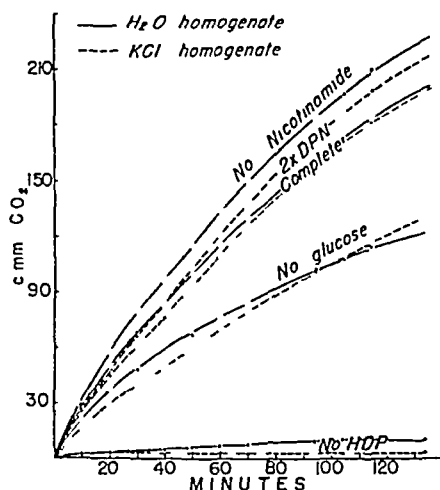


FIG 3 Glycolysis with homogenates of 7 day chick embryos, 100 mg wet weight per flask KCl homogenates compared with water homogenates after freezing and thawing The complete system included 0.008 M HDP, glucose, nicotinamide, DPN, and ATP Oxygen in gas phase

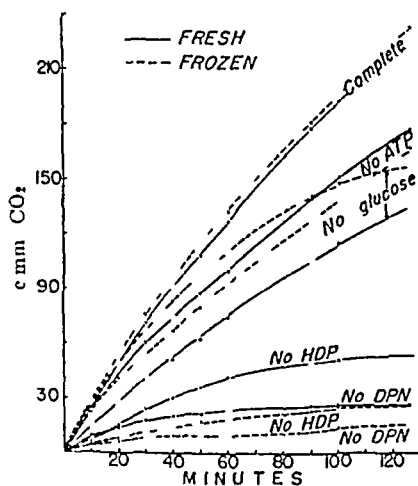


FIG 4 Glycolysis with KCl homogenates of 7 day chick embryos, 100 mg per flask Fresh homogenates compared with frozen homogenates Complete system as in Fig 3 but with 0.004 M HDP and with nicotinamide omitted Oxygen in gas phase

table to the results with KCl homogenates in Fig 1 In addition, the KCl homogenate was tested with the DPN addition doubled The slight increase in activity suggests that the lower level is probably adequate

That freezing the homogenate is unnecessary for obtaining maximum rates of glycolysis in the complete system is shown in Fig 4, in which identical results were obtained with fresh and frozen KCl homogenates, when all of

the reaction components were present. Differences appeared, however, when glucose or HDP were omitted. When glucose was the substrate, the fresh homogenate was superior to the frozen homogenate, and when

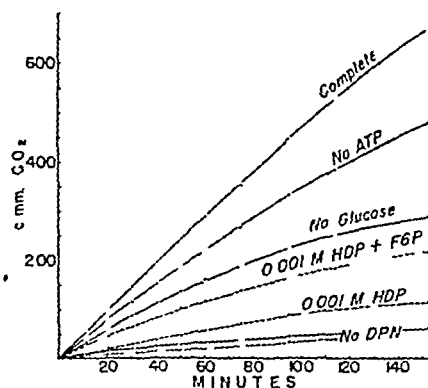


FIG 5 Glycolysis obtained with fresh KCl homogenate of 7 day chick embryos in nitrogen. Complete system includes 0.001 M HDP, glucose, DPN, and ATP, but no nicotinamide. F6P represents fructose 6 phosphate 0.004 M. Solid lines represent 170 mg wet weight of embryo, and broken lines represent 85 mg wet weight.

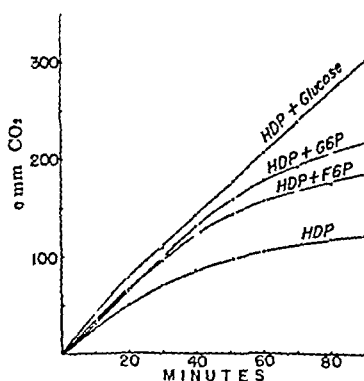


FIG 6 Glycolysis obtained with same homogenate as in Fig 5 but frozen and thawed, and with 114 mg wet weight of embryo per flask. Nitrogen in gas phase, HDP at 0.001 M, glucose at 0.025 M, F6P represents fructose-6-phosphate 0.004 M, G6P represents glucose 6 phosphate 0.004 M. All the flasks contained ATP, DPN, and the basic mixture but no nicotinamide.

HDP was the substrate, the situation was reversed. These results may be explainable in terms of the differences in accessibility of the enzymes in the two homogenates and differences in diffusibility between glucose and HDP.

Figs 5 and 6 show glycolysis by fresh and frozen homogenates in nitrogen, with various substrates. In Fig 5 glucose is shown to be glycolyzed in the presence of 0.004 M HDP and in Fig 6 in the presence of 0.001 M

HDP The increased  $\text{CO}_2$  production obtained with glucose-6-phosphate and fructose-6-phosphate is also shown in Fig 6 With the monophosphates at this critical concentration, the rate is not maintained and falls off as in the case of 0.004 M HDP without glucose (Figs 1 to 5) Glucose at 0.028 M maintains the glycolytic rate better than the monophosphates at 0.004 M when the "priming" level of HDP is present, but is not glycolyzed to any significant extent in the absence of HDP These observations may be explained in terms of the Embden-Meyerhof mechanism by assuming that glucose must be phosphorylated to be glycolyzed

Further experiments with the monophosphates showed an oxygen effect similar to that obtained with glucose and 0.001 M HDP, as shown in Fig 2 This effect was also studied in the case of tumor homogenates and is reported in greater detail elsewhere (20)

TABLE II  
*Lactic Acid Analyses*

	Lactic acid per flask	
	Calculated from $\text{CO}_2$ output	Found
	$\gamma$	$\gamma$
Complete system (HDP, glucose, ATP, DPN, nicotinamide)	2215	2865
Minus nicotinamide	2605	3090
" HDP	881	1255
" glucose	660	838
" HDP and nicotinamide	1384	1640

In only two experiments did we encounter responses different from those described in Figs 1 to 6 Both were with fresh homogenate and in both the oxygen mixture was used In the first a 5 day embryo homogenate gave a sizeable  $\text{CO}_2$  output without any HDP, 76 c mm per 100 mg per 80 minutes, and this was only slightly increased by the addition of HDP In the other case, with a homogenate of 7 day embryos, the  $\text{CO}_2$  output was slightly larger with glucose alone (165 c mm per 165 mg per 200 minutes) than with 0.002 M HDP (120 c mm) When HDP and glucose were present together, the  $\text{CO}_2$  output was much greater than the individual ones combined (150 c mm) Furthermore, in this second case, the omission of DPN or ATP had little or no effect, whether the substrate was HDP, glucose, or both These results are probably related to the findings in Fig 4, and are a reflection of the degree of "intactness" of the enzyme systems in the fresh homogenate

Table II shows the results of chemical analyses for lactic acid for five

of the flasks in this second atypical experiment. A comparison with the amount of lactic acid expected, from the measured  $\text{CO}_2$  output, plus that estimated as having occurred before the zero reading and the lactic acid present in the 165 mg of tissue, shows that the chemical analyses consistently gave higher values.

#### DISCUSSION

The results described point to the conclusion that the typical phosphorylating system of glycolysis exists and functions in the early chick embryo. The phosphorylated intermediates known to take part in the Meyerhof-Embden cycle have been found in 3 to 10 day embryos, with concentrations roughly the same as those found in mature tissues when analyzed in the same fashion, *i.e.*, following freezing in liquid air. Stumpf (29) has recently reported the presence of six of these intermediates in 6 to 10 day chick embryos.

Homogenates of 5 to 8 day embryos have been shown to glycolyze hexose diphosphate, fructose-6-phosphate, and glucose-6-phosphate, in addition to glucose, with the requirements of the glycolyzing system those which would be expected if the Meyerhof-Embden cycle were operative, *i.e.*, DPN is essential, HDP but not glucose is essential, ATP is stimulating. The activity of the homogenates, with only 125 mg per flask, is as high as any shown by Needham and coworkers with very much more tissue, usually 20 ml of undiluted embryo tissue mince.

These results do not disprove the existence of the non-phosphorylating glucolytic pathway postulated by Needham. But there is little, if anything, in them which would indicate its existence. The only possible exceptions are the two experiments in which homogenates of fresh embryos failed to show the typical HDP requirement for glucose glycolysis, and in one of which neither DPN nor ATP omission had any appreciable effect. It might be maintained that our failure to find evidence for the non-phosphorylating mechanism is due to our disruption of the cell structure which Needham and his colleagues claimed to be required for this mechanism. Yet their only evidence for the contention that cell structure is required appears to be their inability to prepare extracts capable of glucolysis.

On the other hand, some of the analytical results we have presented further weaken the evidence which led to the postulation of the non-phosphorylating pathway. We have confirmed Meyerhof and Perdigon's finding (8) that DPN is present in the chick embryo by using the same method of assay which Needham and Lehmann (4) found to give negative results, the malic dehydrogenase system. We have found amounts of ATP and adenylic acid similar to those found in mature tissues where the Meyerhof-Embden cycle is generally considered to operate, despite the

fact that the dry matter of embryo is one-third or one-fourth that in the mature tissues<sup>5</sup> Our demonstration of sizeable quantities of both phosphoglyceric and phosphopyruvic acids indicates that the enzyme needed for the conversion of triose phosphate to phosphoglycerate is not only present, as shown by the manometric data of Meyerhof and Perdigon (8) and those here reported, but active in the living embryo It may be significant that the two embryos (8 and 10 day) with the lowest values for triose phosphate have the highest amounts of phosphoglyceric acids Needham ((30) p 612) has argued, from the marked inhibition of glycolysis by DL-glyceraldehyde, that hexose diphosphate cannot be normally involved in glycolysis because triose phosphate forming from it would remove the DL-glyceraldehyde and limit its inhibition Yet, our analyses demonstrate an unusually high concentration of triose phosphate in the embryo, with some indication, perhaps, of a higher concentration in the 3 to 5 day than in the 8 to 10 day embryos

Thus we are led to the same conclusion which Dorfman (31) reached after reviewing the glycolysis literature through 1942, though it does not rule out the possibility of a non-phosphorylating route of glycolysis, the available evidence does not make necessary postulating the existence of such a route

A few other aspects of the data can only briefly be commented upon

Oxygen has been shown to be markedly inhibitory to the glycolysis of embryo homogenates Yet, in the same system, with only bicarbonate missing, embryo homogenate has an exceedingly small oxygen uptake This would indicate that, for the chick embryo homogenates, the Meyerhof explanation of the Pasteur effect does not apply Needham *et al* (2) reached this same conclusion from studies of the rate of oxidative disappearance of lactic acid The relatively smaller inhibitory effect of oxygen when HDP is being glycolyzed than when glucose is (Figs 1 and 2) might be taken to indicate a point of action of the oxygen between glucose and HDP in the Meyerhof-Embden cycle, as suggested by Engelhardt and Sakov (32)

From the fact that nicotinamide is unnecessary to preserve the DPN activity, it would appear that DPNase is not active in our preparations This is difficult to reconcile with the finding of Meyerhof and Perdigon (8) that chick embryo extract rapidly destroyed DPN We made no attempt to ascertain whether the apparent discrepancy in results stems from the difference in methods of preparing the tissue

We have not tried to account for the consistently higher lactic acid values obtained by chemical analysis than the manometric data would indicate Ochoa (33) found a similar discrepancy in his experiments with brain extracts In his work, corrections of the manometric data were made

<sup>5</sup> Our values for ATP agree quite well with those given by Needham *et al* (2) in terms of easily hydrolyzable phosphorus

for acid changes due to formation or breakdown of hexosephosphoric esters, but not for CO<sub>2</sub> retention by the system. We have made neither correction. In view of the very close parallel between the chemical and manometric data, it seemed safe to use CO<sub>2</sub> liberation as a true measure of glycolysis.

## SUMMARY

1 Chemical analyses of 3 to 10 day chick embryos reveal the presence of glycogen, glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexose diphosphate, triose phosphate, phosphoglyceric acid, phosphopyruvic acid, lactic acid, adenosine triphosphate, adenosine diphosphate, adenylic acid, phosphocreatine, free pentose phosphate, and coenzyme

2 Assay of embryo extract in the malic dehydrogenase system shows the presence of DPN

3 Homogenates of 5 to 8 day embryos have been shown to glycolyze hexose diphosphate, fructose-6-phosphate, glucose-6-phosphate, and glucose. The requirements for the glycolytic system are indicated

4 The bearing of these data on the existence of phosphorylating and non-phosphorylating pathways of glycolysis in the chick embryo is discussed

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# STUDIES ON THE MECHANISM OF THE THROMBOPLASTIC EFFECT\*

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The important rôle in the activation of prothrombin played by a group of macromolecular cytoplasmic lipoproteins, the thromboplastic proteins, has been emphasized in several publications from this laboratory (1-4). The mechanism by which the thromboplastic effect is exerted has, however, remained completely unknown. This is largely due to the difficulty of studying reactions in which the formation of a catalyst is catalyzed, especially when, as is the case in blood clotting, some of the agents and substrates are represented by giant molecules which produce and undergo minute chemical changes.

A beginning should, however, be made by lifting some of the reactions involved out of the sphere of indirect demonstration. This paper presents evidence that the thromboplastic protein is not consumed in the course of the activation of prothrombin and also includes experiments on the preparation of disintegration fragments of this lipoprotein retaining considerable thromboplastic activity and attempts to follow the course of the reaction by spectroscopic observations.

## EXPERIMENTAL

### *Material and Assay Method*

The preparation of the *thromboplastic protein* of beef lung was described previously (5, 6). The specimen used in the course of the present study contained N 70, P 15. In some of the experiments, which will be indicated below, the freshly prepared solution of this substance before its recovery in the dry state was employed. The preparation was entirely free of prothrombin and thrombin.

The dry preparation of *fibrinogen* from human plasma which was used in one set of experiments was obtained through the courtesy of Dr. J. T. Edsall of Harvard University. This preparation was made from blood collected by the American Red Cross under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

\* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.



We are indebted to Dr. W. H. Seegers of Wayne University for a purified sample of cattle *prothrombin* (2000 units per mg. of N).

The assay method which made use of human plasma, centrifuged at a very high speed, has been described in a previous publication (2).

### *Ultraviolet Absorption Spectrum of Thromboplastic Protein*

The thromboplastic protein of beef lung was caused to sediment by centrifugation at 31,000*g*, following purification in several centrifugal cycles (6), and the pellets were taken up in 0.1 M borate buffer of pH 8.3. The solution was once more subjected to centrifugation at 5000*g* for 30 minutes. Analysis of aliquots of the supernatant showed the presence of 4.28 mg. of the thromboplastic protein per cc. of solution. For the spectroscopic observations in a Beckman photoelectric quartz spectrophotometer the freshly prepared solution was diluted 1:100 with borate buffer. The absorption spectrum is reproduced in Fig. 1. When the solution was allowed to stand, even at 4°, the maximum at 227 m $\mu$  gradually disappeared, with the development of a continuously increasing end-absorption, the plateau between 270 and 260 m $\mu$  however, persisted. Indications were obtained that this spectral change on storage was caused by the gradual aggregation of the protein in solution, since the original spectrum could sometimes be restored by the centrifugation of such solutions at 5000*g*.

The total lipides isolated from a portion of the thromboplastic protein, when examined in chloroform solution, exhibited an absorption spectrum which, in the region between 300 and 250 m $\mu$ , resembled that given by the intact protein. They showed a slight maximum at 275 m $\mu$  (specific extinction  $k = 0.76$ ) and a minimum at 263 m $\mu$  ( $k = 0.72$ ). For another lipid preparation the  $k$  values at the same wave-lengths were 0.69 and 0.66 respectively. The treatment of the lipid solution with 0.1 M sodium bisulfite produced no change in the spectrum. The aqueous solution of the bases recovered from an acid hydrolysate of the lipid preparation exhibited no selective absorption in the ultraviolet.

### *Rate of Thromboplastic Protein Following Prothrombin Activation*

*Recovery Experiments*—In preliminary experiments, as described previously (6), it was found that about 1  $\gamma$  of thromboplastic protein was required for the complete activation of 15  $\gamma$  of the prothrombin sample within 20 minutes at 30°.

To a solution of 80 mg. of prothrombin in 5 cc. of borate buffer containing 150 mg. of calcium nitrate (pH 7.7) 1 cc. of about 0.4 per cent solution of the thromboplastic protein in borate buffer of pH 8 was added. A control experiment in which prothrombin was omitted, was carried out through all operations. The mixture was kept at room temperature for 1 hour in a

closed lusteroid tube and then subjected to centrifugation at 31,000*g* for 90 minutes. The supernatant proved, of course, extremely rich in thrombin. The translucent pellet was dispersed in 8 cc of borate buffer of pH 8 and the solution again centrifuged at 31,000*g* for 90 minutes. This procedure

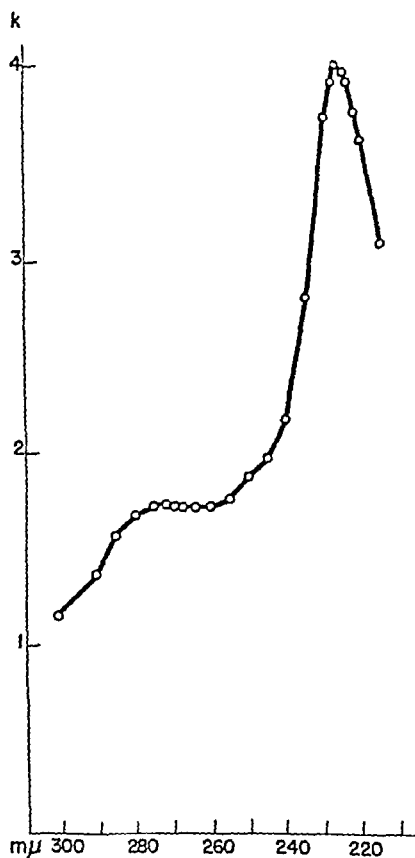


FIG 1 Ultraviolet absorption of thromboplastic protein of beef lung in 0.1 M borate buffer of pH 8.3 (0.0428 gm per liter).  $k$ , the specific extinction, =  $E/cd$ , where  $E = \log I_0/I$ ,  $c$  = concentration in gm per liter,  $d$  = thickness of absorbing layer in cm.

was repeated once more.<sup>1</sup> The solution of the sediment was dialyzed for 24 hours against running tap water and for 48 hours against ice-cold distilled water. The protein, recovered by the evaporation of the water from the frozen solution *in vacuo*, weighed 2.9 mg (Fraction Tp-P). The corresponding fraction (Tp-C), recovered in the control experiment, weighed 3.0 mg.

<sup>1</sup> The adequacy of the washing was controlled by thrombin tests.

These fractions were assayed for thromboplastic activity as shown in Table I. The results were surprising: the thromboplastic protein fraction recovered after the activation of prothrombin had not only retained its activity, it actually was more potent.

*Spectroscopic Observations*—In the course of the recovery study presented in the preceding paragraphs samples were removed from both the prothrombin and the control experiments at various stages of the purification of the thromboplastic protein and examined spectroscopically. No essential change in the shape of the spectrum reproduced in Fig. 1 was observed.

TABLE I

*Activity of Thromboplastic Protein before and after Activation of Prothrombin*

The experiments were carried out at 37° by mixing 0.1 cc. of plasma with 0.1 cc. of the saline suspensions of the thromboplastic protein and 0.2 cc. of a 0.01 M  $\text{Ca}(\text{NO}_3)_2$  solution (containing 0.4 per cent of NaCl). Tp represents the preparations used for the experiments, Tp-P the fraction recovered after the activation of prothrombin, Tp-C the fraction recovered in the control experiment.

Preparation	Concentration in experiment	Clotting time	
		Plasma 1	Plasma 2
	$\gamma$	sec	sec
Tp	100	63	71
	10	85	90
	1	128	132
Tp-P	100	18*	20
	10	70	52
	1	111	78
Tp-C	100	77	90
	10	130	145
	1	188	222

\* The virtual absence of thrombin from this preparation is demonstrated by the fact that, when  $\text{Ca}^{++}$  was omitted, a slow deposition of fibers started only after 360 seconds.

The center of absorption at 227  $\text{m}\mu$  had become obliterated in all samples, but this, as was pointed out before, also took place with untreated stored solutions. The significance of one other observation cannot yet be determined: although the concentrations were kept as nearly identical as possible in both experiments, the samples recovered after the activation of prothrombin exhibited consistently higher extinction values (30 to 70 per cent at different stages of the experiment) than did the control samples.

Another type of experiment is exemplified in Fig. 2. The spectra (in the region of 300 to 240  $\text{m}\mu$ ) of the thromboplastic protein (Curve I) and of the prothrombin<sup>2</sup> (Curve II) were compared with the spectrum given by a mix-

\* Ultraviolet absorption spectra of more highly purified prothrombin have been published by Seegers *et al.* (7).

ture of these components (Curve III) which, since calcium ion was present in all solutions, corresponded essentially to thrombin. If the extinction

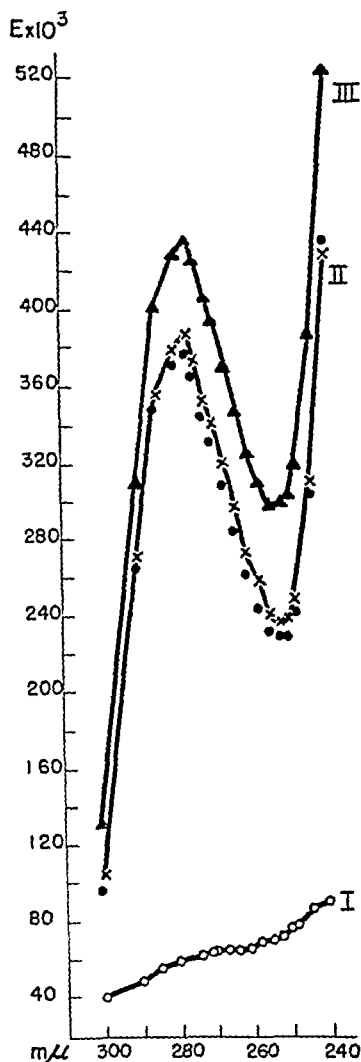


FIG 2 Ultraviolet absorption of mixtures of thromboplastic protein and prothrombin. All experiments were carried out in 1 cm cells in 0.1 M borate buffer of pH 8.3 containing 900  $\gamma$  of  $\text{Ca}(\text{NO}_3)_2$  per cc. Curve I, 40  $\gamma$  of thromboplastic protein per cc; Curve II, 300  $\gamma$  of prothrombin per cc; Curve III, 40  $\gamma$  of thromboplastic protein and 300  $\gamma$  of prothrombin per cc. The mixtures were kept at room temperature for 30 minutes before spectroscopy. The solid dots indicate the differences in the extinction values plotted as Curves I and III.

values for Curve I are subtracted from those of Curve III, i.e. if allowance is made for the unchanged properties of the thromboplastic protein during

activation, a new curve can be constructed (likewise indicated in Fig 2) which in all essentials corresponds to Curve II, the spectrum of the prothrombin solution. This experiment, therefore, likewise failed to reveal a spectral change during activation. It should be emphasized that these observations, because of the contamination of the prothrombin employed with other plasma globulins, have only qualitative validity.

### *Influence of Fibrin Formation on Activity of Thromboplastic Protein*

This experiment was designed to test the fate of the thromboplastic protein in the course of the coagulation of fibrinogen. 60 cc of the fresh 0.43 per cent solution of the thromboplastic protein in borate buffer of pH 8.3 were mixed with 15 cc of a solution of the human fibrinogen powder containing a total of 210 mg of fibrinogen. 65 cc of this mixture (containing about 224 mg of the thromboplastic protein, 182 mg of fibrinogen) were centrifuged for 30 minutes at 5000*g*. The supernatant was subjected to centrifugation at 31,000*g* for 90 minutes and this was repeated with the suspension of the sediment in 65 cc of borate buffer of pH 8.3. (At this stage the supernatant failed to show the presence of fibrinogen when tested with thrombin.) To the suspension of the pellets in 30 cc of borate of pH 8.3 a solution of 10 mg of thrombin<sup>2</sup> in 5 cc of the same buffer was added and the mixture was kept for 30 minutes at room temperature and for 15 hours in the refrigerator. The thromboplastic protein was then again precipitated by centrifugation at 31,000*g* for 90 minutes. The pellets, which consisted of an elastic, difficultly dispersible material, were washed with borate buffer under the same centrifugal conditions, resuspended, dialyzed against running and ice-cold distilled water, and recovered by evaporation of the frozen solution in a vacuum. *Fraction Tp-F*, 89.5 mg of a white felt, contained N 7.6, P 1.5. When assayed for thromboplastic activity, as described in Table I, the clotting times (in seconds) for two plasma samples were as follows: 100  $\gamma$ , 30, 30, 10  $\gamma$ , 50, 59, 1  $\gamma$ , 84, 91.

### *Effect on Thromboplastic Protein of Strong Sodium Chloride, Guanidine Hydrochloride, and Sodium Desoxycholate*

In these experiments, which are presented in detail in Table II, 0.2 per cent dispersions of the dry thromboplastic protein preparation in M sodium chloride, 5 M guanidine hydrochloride, and 0.012 M sodium desoxycholate were stored for 24 hours in the refrigerator. The mixtures were centrifuged for 90 minutes at 31,000*g* and the pellets suspended in borate buffer of pH 7.7. These suspensions and the supernatants were dialyzed for 68 hours

<sup>2</sup> We are indebted to Dr. D. W. MacCorquodale of the Abbott Laboratories, North Chicago, Illinois, for this preparation.

against running tap water, for 72 hours against ice-cold distilled water, and then concentrated in a vacuum in the frozen state

The various fractions were assayed for thromboplastic activity. In most instances their lipid contents and the composition of the lipid-free residues were also determined by a procedure previously described (3) (see Table II)

TABLE II  
*Disintegration of Thromboplastic Protein by Various Agents*

Experiment No	Agent	Disintegration products							Thrombo- plastic activity*	Lipide free residue		
		Distribution	Pro- por- tion of start- ing mate- rial	N		P		N		P	P	
					Pro- por- tion of start- ing mate- rial		Pro- por- tion of start- ing mate- rial					
			per cent	per cent	per cent	per cent	per cent	sec	per cent of original fraction	per cent	per cent	
1	None		100	7 0	100	1 5	100	71 90	48	13 1	0 65	
2	0.012 M sodium chlo- ride, pH 6.5	Sediment	80 3	7 1	81 5	1 1	58 9	112 148	43	11 6	0 5	
		Supernatant	9 3	7 1	9 4	0 7	4 3	88 110				
3	5 M guanidine hydrochlo- ride, pH 6.9	Sediment	64 8	6 1	56 5	1 3	56 1	85 114	34	10 1	0 7	
		Supernatant	31 2	8 7	38 8	0 6	12 5	Inac- tive	56		0 4	
4	0.012 M sodium desoxycho- late, pH 7.3	Sediment	31 7	8 5	38 5	0 7	14 8	"	51	13 2	0 3	
		Supernatant	67 6	4 9	47 3	1 0	45 1	70 91	37	10 7	0 6	

\* Clotting times observed with 100  $\gamma$  and 10  $\gamma$  respectively of the test substances in the arrangement described in Table I

#### DISCUSSION

The possible effects of the thromboplastic protein can be reduced to the following propositions: (a) it catalyzes the activation of prothrombin without itself entering into the reaction (8), (b) it (or one of its components) combines with the prothrombin to produce thrombin (9), (c) it removes a portion of the prothrombin (perhaps a thrombin inhibitor) and thereby releases the active thrombin (10). The first statement implies an essentially enzymatic function, and this has been expressed in the often used term thrombokinase. The second choice involves the consumption of the thromboplastic protein in the course of a stoichiometric reaction. The same is true of the third possibility listed, unless the removal of the inhibitor is postulated as an enzymatic reaction, in which case propositions (a) and (c)

become identical. Among the studies supporting the non-enzymatic character of the thromboplastic agent that of Meitz *et al* (11) should be mentioned.

Some of the experiments presented in this paper, however, lend strong support to the conception of the enzymatic nature of the tissue factor. They were based on the fortunate fact that the thromboplastic protein, because of its centrifugal characteristics, can be isolated from the reaction mixture by centrifugation at high speed following its action on prothrombin. The recovered thromboplastic protein had not lost its activity, it had, in fact (and this cannot yet be explained) somewhat increased in potency.<sup>4</sup> If the conditions under which the experiments showing a proportionality between the amount of thrombin produced from a prothrombin excess and the amount of thromboplastin present were conducted (11) are comparable to the experimental arrangement reported here, it may be concluded that the catalytic activation of prothrombin takes place under equilibrium conditions in which an excess of substrate (prothrombin) is inhibiting. This would serve to classify the thromboplastic effect as a reaction with explosive tendencies proceeding via branched chains (12). The spectroscopic evidence submitted here, although not contributing to a deeper insight into the reaction mechanism, also favors the view that the thromboplastic protein remains unchanged during the reaction.

When the coagulation of fibrinogen takes place under physiological conditions, the thromboplastic agent which acted as the initiator presumably still is present. It was, therefore, of interest to ascertain whether the thromboplastic protein, caused to sediment from a fibrinogen solution and then treated with thrombin, in order to coagulate whatever fibrinogen had been adsorbed, was inactivated or had retained full activity. The latter has now been found to be the case.

The experiments on the disintegration of the thromboplastic protein by various chemical agents require some comment. Since this lipoprotein is perhaps the only representative of this group whose biological activity can be followed with ease, the study of the chemical alterations to which it can be subjected without complete inactivation may be of more general instructiveness. In a recent study from this laboratory (2) it has been shown that practically the entire thromboplastic activity of aqueous lung extracts was confined to one fraction sedimentable at a high centrifugal speed. Even when the partial disintegration of the lipoprotein was brought about

<sup>4</sup> It could be argued that even if the reaction between the thromboplastic protein and prothrombin were non-enzymatic any unused portion of the thromboplastic agent would be expected to be recoverable by centrifugation. But this is made extremely unlikely by the results of the control experiment and the parallel spectroscopic observations.

by freezing in the presence of ether, whatever activity remained went into the sediment (6, 13)

The effects obtained with strong sodium chloride and with two agents known to denature proteins, *viz* guanidine and sodium desoxycholate, may be summarized as follows. They all attack the thromboplastic protein, the first effect shown by all agents being the detachment of some phosphorylated compounds in such a manner as to render them dialyzable. As will be seen in Table II, after treatment with  $\approx$  NaCl about 91 per cent of the nitrogen of the starting material was recovered, but only 63 per cent of the phosphorus, the corresponding figures for guanidine were 95 and 69 per cent, for sodium desoxycholate 86 and 56 per cent. Otherwise, as might have been expected, strong sodium chloride was the least effective of the agents studied. Guanidine cleaved the thromboplastic protein, rendering about one-third non-sedimentable and inactive, the sedimentable part retained activity. Sodium desoxycholate had the most interesting effect: it detached about two-thirds of the protein in a non-sedimentable form retaining full thromboplastic activity. This is the first instance of this kind observed in our studies and may be of value for future work.

The assistance of Mrs. Charlotte Green is gratefully acknowledged.

#### SUMMARY

The thromboplastic protein, following its reaction with prothrombin, can be recovered, by high speed centrifugation, with full activity. This finding lends strong support to the conception of the enzymatic nature of the thromboplastic agent.

The thromboplastic protein, when caused to sediment from a fibrinogen solution and treated with thrombin, retains full activity.

Guanidine, sodium desoxycholate, and to a lesser degree strong sodium chloride, have a far reaching effect on this lipoprotein. Of particular interest is the action of sodium desoxycholate, with the aid of which it has for the first time been possible to separate a non-sedimentable disintegration fragment with considerable thromboplastic activity.

The ultraviolet absorption spectrum of the thromboplastic protein is reported and spectroscopic observations and general remarks on the mechanism of the thromboplastic effect are included.

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# ON THE INHIBITION OF THE THROMBOPLASTIC EFFECT\*

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The search for inhibitors of the thromboplastic protein, apart from theoretical deductions to which it may lead, may also be of practical interest, if, as was recently shown to be probable (1), minute quantities of substances of this kind are present in circulating blood. An increased excretion of the thromboplastic protein following cellular damage could be expected to lead to the formation of the thrombi.<sup>1</sup>

The inhibition of the thromboplastic effect involves the first phase of blood coagulation, the preparatory step in which prothrombin is activated to thrombin, but to decide whether a particular inhibitor acts on the thromboplastic protein or on the prothrombin is extremely difficult. These considerations do not apply, of course, to agents, such as dicumarol, that prevent the production of prothrombin *in vivo*.

This paper presents the results of a study of the action of a number of inorganic and organic compounds on the thromboplastic effect, examined in as simple a system as possible. The experimental arrangement was so designed as to permit the distinction between the inhibition of thrombin formation and that of thrombin action. The concentration of the inhibitor was the only variable, the amounts of thromboplastic protein, prothrombin, and fibrinogen (which all were employed as purified preparations) being kept constant. This voluntary limitation precluded the examination of the various factors known or claimed, to occur in nature that are commonly designated, though by no means authenticated, by the prefix anti. The important anticoagulant heparin also was excluded for the present, since the required addition of the heparin complement would have complicated the experimental system.

The action of metal salts on the coagulation of blood has been studied by several authors (*e g* (2-7)), but the experimental conditions under which the results were obtained are open to serious objections. In many instances no attention was paid to the hydrogen ion concentration of the clotting system, in almost all cases whole blood or plasma was used, which not only

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<sup>1</sup> A method similar to the one outlined in Table III of a recent publication (1) could conceivably serve as a basis for the estimation of circulating thromboplastic protein.

complicated the interpretation of the findings, because of potential reactions between the metals and other blood proteins, but also made difficult the differentiation between the inhibitions of the thromboplastic and of the thrombic effects, and in no case was the establishment of a standardized incubation time of the thromboplastic agent or the prothrombin with the inhibitor possible

The results obtained in the present study are summarized in Table I. It will be seen that of the metals inhibiting the first phase of coagulation only, *i.e.* the formation of thrombin, *scandium*, *yttrium*, and *lanthanum* were by far the most active.<sup>2</sup> This is in harmony with the known anticoagulant properties of rare earth salts, both *in vivo* and *in vitro* (2, 4-7). With the exception of scandium, of which little appears to be known (8, 9), these trivalent cations seem, however, too toxic for practical application (9-13), though vitamin K has been reported to overcome the lowering by rare earth salts of the prothrombin level in animals (14).

The effect of lanthanum acetate on the thromboplastic protein was studied in greater detail. This salt was found to bring about the flocculation of the protein, so as to render it precipitable in a much weaker centrifugal field than must be employed normally. This effect is probably due to the well known precipitating action of lanthanum salts on nucleic acids and nucleoproteins (15, 16).<sup>3</sup> There appears, however, to be no connection with the anticoagulant properties discussed here, since the thromboplastic protein, following its precipitation by La, showed undiminished activity. The evidence, submitted in the experimental part (compare, *e.g.*, Table II), favors, in fact, the assumption that the inhibition of the thromboplastic effect by rare earth salts derives from their reaction with prothrombin. It is possible, though not likely, that this has to do with the displacement of the calcium ions necessary for the formation of thrombin.

The only non-metallic compound that showed a very appreciable inhibiting effect was *sodium desoxycholate*. The action of this substance on the thromboplastic protein has been discussed in the preceding publication (17).

<sup>2</sup> It must, however, be understood that, because of the varying degrees of basicity and hydrolysis, the quantitative evaluation of biological metal effects meets with great difficulties.

<sup>3</sup> It should be mentioned that the formation of very insoluble nucleic acid salts does not only pertain, as would be expected, to other trivalent cations of the same group, such as scandium and yttrium, but also to the trivalent complex cation  $(\text{Co}(\text{NH}_3)_6)^{+++}$ . There is, however, one important difference which might acquire interest: whereas the rare earths also precipitate nucleotides, the luteo salt hexammine cobaltic chloride does not do so.

## EXPERIMENTAL

*Material*

The *fibrinogen* used in these studies was prepared from human plasma in the Department of Physical Chemistry, Harvard Medical School, and placed at our disposal through the kindness of Dr J T Edsall. It was obtained as a dry powder containing about 46 per cent of fibrinogen, 16 per cent of other plasma proteins, and 38 per cent of sodium citrate. Aqueous solutions of this powder were used in the experiments.

The *prothrombin* used was prepared from beef plasma in the laboratories of Parke, Davis and Company, Detroit, and furnished to us by Dr W H Seegers, now of Wayne University. We wish to thank Dr Seegers for this preparation which was obtained as a dry powder containing 2000 units per mg of nitrogen.

Two preparations of the *thromboplastic protein* of beef lung were employed in the present work. One has been referred to in the preceding paper (17), the other, similarly prepared, had a slightly higher activity and contained N 8.0, P 1.7 per cent.

The various compounds tested for inhibitor action were commercial preparations with the exception of the following substances which were prepared in this laboratory. *Scandium chloride*,  $\text{ScCl}_3$ , was made from scandium oxalate<sup>4</sup> (calculated for  $\text{Sc}_2\text{C}_2\text{O}_4 \cdot 5\text{H}_2\text{O}$ , Sc 20.3, found, Sc 20.4) via the oxide  $\text{Sc}_2\text{O}_3$ . *Yttrium acetate*,  $\text{Y}(\text{C}_2\text{H}_3\text{O}_2)_3 \cdot 4\text{H}_2\text{O}$ , was prepared from the commercial oxide  $\text{Y}_2\text{O}_3$  via the hydroxide  $\text{Y}(\text{OH})_3$ , which was precipitated with ammonia in the presence of ammonium tartrate in order to exclude the cerium earths (18). The luteo salt, *hexammine cobalt chloride* ( $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ ), was prepared from  $\text{CoCl}_2$  by oxidation with an ammoniacal silver chloride solution (19), the purpureo salt, *chloropentammine cobalt chloride* ( $\text{Co}(\text{NH}_3)_5\text{ClCl}_2$ ), was made from  $\text{CoCO}_3$  by the method of Sørensen (20) (compare also (21)). The *acetyl acetone complex of lanthanum*,  $\text{La}(\text{C}_5\text{H}_7\text{O}_2)_3$ , was obtained from lanthanum nitrate (22, 23).

The buffers used were veronal buffers (24) and 0.1 M acetate buffers with the pH values indicated in Tables I and II.

*Assay Methods*

*Antithromboplastic Effect (Method A)*—Mixture 1 contained 0.5 cc of a 0.04 per cent solution of the thromboplastic protein and 0.5 cc of a 0.05 M solution of the inhibitor in buffer and was kept for 30 minutes at room temperature. Of those metal salts that proved inhibiting, serial dilutions

<sup>4</sup> We are greatly indebted to Dr S H Hutner, Haskins Laboratories, New York, for this compound.

of the 0.05 M solution (0.005 M, etc.) were likewise tested. In *Mixture 2*, 0.1 cc. of a 0.1 per cent prothrombin solution in buffer, which was made 6 mM with respect to calcium nitrate, was added to 0.1 cc. of *Mixture 1*, and the solution was kept for 20 minutes at room temperature. *Mixture 3*, employed in the clotting tests carried out at 30°, was obtained by adding 0.2 cc. of a 1.4 per cent<sup>5</sup> fibrinogen solution (pH 6.45) to 0.03 cc. of *Mixture 2*. With 0.05 M inhibitor solutions, *Mixture 1*, therefore, contained in 1 cc. 200  $\gamma$  of the thromboplastic protein and 25 micromoles of the inhibitor, *Mixture 2*, 100  $\gamma$  of the thromboplastic protein, 500  $\gamma$  of prothrombin, 12.5 micromoles of the inhibitor, and 3 micromoles of calcium.

The solutions were all freshly prepared and never stored for more than a few hours at 0°. The control experiments, carried out each day with the omission of the inhibitor, gave clotting times of 15 to 20 seconds.

Much attention was paid to the maintenance of a constant hydrogen ion concentration during the inhibition experiments. As will be seen in Table I, the pII values of the solutions of the metal salts as well as those of the inhibitor-thromboplastic protein mixtures (*Mixture 1*) have been recorded. In many cases the pH of *Mixture 2*, which never differed much from that of *Mixture 1*, and of *Mixture 3*, which was found to be around pH 6.2 to 6.4, was also determined.

*Antithrombic Effect (Method B)*—A mixture of 0.1 cc. of a 0.1 per cent prothrombin solution (6 mM with respect to calcium nitrate) and of 0.1 cc. of a 0.02 per cent solution of the thromboplastic protein was kept for 20 minutes at room temperature. For the clotting tests (at 30°), 0.03 cc. of this solution was mixed with 0.03 cc. of the 0.05 M inhibitor solution, and 0.2 cc. of a 1.4 per cent fibrinogen solution was added. The effects are described in Table I.

### *Influence of Various Agents on Thromboplastic Effect*

The following substances were without effect on the thromboplastic protein when tested in 0.05 M solution in veronal buffer by assay Method A: sodium arsenite (pII 8.3), sodium cyanide, sodium fluoride, sodium azide, sodium iodate, sodium persulfate, iodine (in KI), hydroxylamine (pII 7.0 to 7.4).

The only substance that was found inhibiting, apart from the metal salts listed in Table I, was *sodium desoxycholate*. When tested in veronal buffer (pH 7.5) by Method A, the results were as follows: 0.05 M + + +, 0.017 M + +, 0.006 M  $\pm$ , 0.002 M —. No antithrombic effect was observed (Method B).

<sup>5</sup> This figure is based on the amount of coagulable fibrinogen present in the protein powder.

TABLE I  
Inhibition of Thromboplastic Effect by Metals

Group No in periodic system	Cation*	Buffer	pH of inhibitor solution	pH of inhibitor thrombo- plastic protein (Mixture 1)†	Assay method†	Inhibition‡ Molarity of inhibitor solution used			
						0.05 mM	0.005 mM	0.0005 mM	0.0001 mM
I	Cu <sup>++</sup>	Acetate	6.0	6.3	A	+++	+++	+++	±
					B	±	—	—	—
	Ag <sup>+</sup>	"	6.2	6.2	A	+++	+++	++	—
					B	±	—	—	—
II	Zn <sup>++</sup>	"	6.9	6.2	A	+++	—	±	—
					B	—	—	—	—
	Cd <sup>++</sup>	Veronal	7.0	7.0	A	+++	—	—	—
					B	—	—	—	—
III	Sc <sup>+++</sup>	Acetate	6.6	6.5	A	+++	+++	+++	+++
					B	—	—	—	—
	Y <sup>+++</sup>	Veronal	5.9	5.8	A	+++	—	+++	±
					B	—	—	—	—
	La <sup>+++</sup>	Acetate	6.3	6.2	A	+++	—	+++	—
					B	—	—	—	—
	Nd <sup>+++</sup>	"	5.8	6.0	A	+++	—	+	±
					B	—	—	—	—
IV	Ti <sup>+</sup>	"	7.0	6.3	A	—	—	—	—
					B	—	—	—	—
	Ce <sup>+++</sup>	"	7.3‡	6.4	A	+++	+++	±	—
					B	±	—	—	—
	Pb <sup>++</sup>	"	6.6	6.6	A	+++	—	+	—
					B	—	—	—	—
	Th <sup>++++</sup>	"	6.7‡	6.6	A	+++	+++	—	—
					B	+	±	—	—
VI	Cr <sup>+++</sup>	"	6.6‡	6.4	A	+++	—	—	—
					B	—	—	—	—
	U <sup>+++</sup>	"	5.9‡	6.1	A	+++	+++	+++	—
					B	±	—	—	—
VII	Mn <sup>++</sup>	Veronal	6.6	6.6	A	—	—	—	—
VIII	Co <sup>++</sup>	"	7.0	6.8	"	+++	—	—	—
	(Co(NH <sub>2</sub> ) <sub>6</sub> ) <sup>+++</sup>	"	7.2	6.9	A	±	—	—	—
	(Co(NH <sub>2</sub> ) <sub>5</sub> Cl) <sup>++</sup>	"	6.8	6.7	B	—	—	—	—
					A	—§	—	—	—

\* The metals of Group I were tested as the acetates, those of Groups VII and VIII as the chlorides. Cd, Y, La, Pb were acetates, Zn, Sc, Nd, Ce were chlorides, Th and Cr were nitrates. Ti was tested as thallous formate, U as uranyl acetate.

† See the experimental part for a description of the methods of assay. Inhibition is classified as follows: +++ no clot within 180 seconds, ++ clot between 120 and 180 seconds, + clot between 60 and 120 seconds, ± clot between 30 and 60 seconds, — no inhibition (clotting time as in the control).

‡ These solutions were not entirely stable and deposited a precipitate.

§ Because of the limited solubility of this complex, a 0.01 M solution was employed.

*Attempts at Reversal of Metal Inhibition*

*Copper*—A mixture of 1 cc of a 0.1 M solution of cupric acetate and 1 cc of a 0.08 per cent solution of the thromboplastic protein (acetate buffer, pH 6.0) was kept at room temperature for 30 minutes. To 0.4 cc aliquots of this solution 0.4 cc portions of either acetate buffer, 0.1 M sodium cyanide, or 0.12 M sodium cyanide were added and each of the three mixtures was permitted to act on a 0.1 per cent prothrombin solution (in the presence of calcium), as described above. The copper effect was not annulled by NaCN, the inhibition corresponded to +++ (compare Table I).

*Silver*—When 0.08 cc of a 0.4 M sodium chloride solution was added to 0.8 cc of a mixture of equal parts of 0.05 M silver acetate and 0.04 per cent thromboplastic protein (acetate buffer, pH 6.2), following incubation for 30 minutes, the inhibition of thrombin formation was decreased from +++ to + (observed clotting times 64 and 69 seconds).

*Lanthanum*—The inhibition of a 0.05 M lanthanum acetate solution (see Table I) was not reversed by the addition of 4 equivalents of acetylacetone to the inhibitor-thromboplastic protein mixture. Acetylacetone itself was without effect on the clotting time. The preformed lanthanum-acetylacetone complex, employed as a suspension in buffer, was found inhibiting.

It should be mentioned that the usual +++ inhibition was observed when the thromboplastic protein, lanthanum acetate, prothrombin, and calcium nitrate were incubated together instead of in two steps, as is normally done.

*Influence of Large Excesses of Clotting Agents on Lanthanum Inhibition*

The studies described so far were carried out with constant amounts of thromboplastic protein and prothrombin. It appeared of interest to study the influence of large excesses of these agents on the inhibitory effect of lanthanum acetate which was employed in a fixed, very low concentration. The results, given in Table II, show that the inhibition could be only partially reversed by an excess of thromboplastic protein (Experiments 3 and 4), but completely abolished by a large quantity of prothrombin (Experiment 5).

*Study of Lanthanum Effect*

A freshly made solution of the purified thromboplastic protein of beef lung (25) in borate buffer of pH 8.3 was subjected to centrifugation at 31,000*g* for 2 hours and the pellets were taken up in 0.1 M acetate buffer of pH 6.3 to yield an approximately 0.35 per cent solution. One 15 cc aliquot served as control. To another 15 cc portion 5 cc of a 0.05 M solution of lanthanum acetate in 0.1 M acetate buffer (pH 6.3) were added, whereupon

an almost immediate fine precipitation occurred. Both mixtures were centrifuged for 30 minutes at 5000g. The supernatant from the control experiment was strongly opalescent,<sup>6</sup> whereas that of the preparation treated with La was clear.

The sediment obtained in the presence of lanthanum was washed at 5000g with acetate buffer containing 0.1 per cent of lanthanum acetate and several times with distilled water. Its aqueous suspension was frozen and evaporated in a vacuum, when 50.9 mg of a colorless felt were obtained. It contained N 6.8, P 1.5 per cent, the lipid-free residue (compare (17)) amounted to 44 per cent of the total. The supernatant of this fraction, dialyzed and concentrated in the usual manner, yielded no weighable residue.

TABLE II

*Influence of Large Excesses of Clotting Agents on Lanthanum Inhibition*

Experiment No	Composition of inhibition mixture * per cc				Clotting time
	Thromboplastic protein	Lanthanum acetate	Prothrombin	Calcium nitrate	
	$\gamma$	$\mu$ mole	$\gamma$	$\mu$ moles	sec
1	100	0	500	3	16
2	100	0.125	500	3	63
3	200	0.125	500	3	50
4	1000	0.125	500	3	29
5	100	0.125	5000	3	12

\* Acetate buffer (0.1 M) served as the solvent, the pH of the experiments was 6.1. For the clotting time determinations, 0.2 cc of a 1.4 per cent fibrinogen solution was added to 0.03 cc of the inhibition mixtures, following their incubation for 30 minutes at room temperature.

The supernatant of the control experiment (with the omission of lanthanum), on the other hand, yielded 34 mg of unchanged thromboplastic protein containing N 7.0, P 1.4 per cent.

The thromboplastic activities of both fractions, assayed as described in the preceding paper (17), were found very similar. The clotting times (in seconds) for decreasing amounts of the fraction precipitated by lanthanum were as follows: 100  $\gamma$ , 63, 10  $\gamma$ , 85, 1  $\gamma$ , 150. The corresponding figures for the preparation recovered in the control experiment were as follows: 100  $\gamma$ , 61, 10  $\gamma$ , 96, 1  $\gamma$ , 170. The fraction precipitated by La could still be inhibited by lanthanum acetate when tested in the regular manner.

<sup>6</sup> At pH 6.3 some thromboplastic protein sedimented at 5000g, probably because of aggregation. This has usually not been observed at higher pH values.



## SUMMARY

The inhibition of the thromboplastic effect has been studied. The trivalent cations scandium, yttrium, and lanthanum have been shown to be the most potent inhibitors. Sodium desoxycholate also had a very appreciable activity. The mechanism of the inhibition is discussed.

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## STEROIDS DERIVED FROM BILE ACIDS

### VI AN IMPROVED SYNTHESIS OF METHYL 3,9 EPOXY- $\Delta^{11}$ CHOLENATE FROM DESOXYCHOLIC ACID\*

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A method has been described for the preparation of 3( $\alpha$ )-hydroxy-11-ketocholanic acid in which methyl 3,9-epoxy- $\Delta^{11}$ -cholelate is an essential intermediate (1). The steps from desoxycholic acid through the 12-benzoate and the  $\Delta^{11}$ -cholelic acid are shown in the left column of Fig 1.

Two of the steps in this series of reactions are not satisfactory for large scale production. (1) The introduction of a double bond at C<sub>11</sub>-C<sub>12</sub> through pyrolysis of the 12-benzoate leaves much to be desired in respect to both the labor required and the yield. (2) Conversion of 3( $\alpha$ )-hydroxy-11,12-dibromocholanic acid to 3( $\alpha$ ),12-dihydroxy- $\Delta^9$  <sup>11</sup>-cholelic acid is readily accomplished but the yield is poor. An improved method for the synthesis of methyl 3,9-epoxy- $\Delta^{11}$ -cholelate has now been found which eliminates both of these unsatisfactory steps. The procedure is outlined in the column on the right side of Fig 1.

Commercial desoxycholic acid was partially purified by thorough extraction with boiling benzene<sup>1</sup> and subsequent crystallization from acetone-water, 4:1. It was then esterified at room temperature in methanol in the presence of 0.1 N hydrochloric acid and the ester was treated in benzene with benzoyl chloride and pyridine to obtain methyl 3-benzoyldesoxycholate.

The 12-hydroxyl group of methyl 3-benzoyldesoxycholate was oxidized to a ketone with chromic acid in a mixture of chlorobenzene-acetic acid, 4:1, to obtain methyl 3( $\alpha$ ) benzoy-12-ketocholanoate (II).<sup>2</sup>

The double bond was introduced into Ring C with selenium dioxide. Schwenk and Stahl (3) first observed that this reagent would form the  $\Delta^9$  <sup>11</sup> compound from methyl 3( $\alpha$ )-acetox-12-ketocholanoate. A detailed study in this laboratory of the dehydrogenation of methyl 3( $\alpha$ )-benzoy-12-ketocholanoate with selenium dioxide in chlorobenzene and acetic acid (4:1) as the solvent has been carried out. The influence of time and a small

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<sup>1</sup> Extraction of fatty acid with benzene has been found effective and more convenient than with xylene, which has been used by White (2).

<sup>2</sup> The roman numerals refer to the structural formulas in Fig 1.

amount of hydrogen chloride on the yield of the unsaturated compound is shown in Table I. Refluxing for 24 hours without hydrogen chloride and for 72 hours with 0.0006 N hydrogen chloride gave respectively 67 and 84 per cent of 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9,^{11}$ -cholanic acid.

Seebeck and Reichstein (4) have shown that 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9,^{11}$ -cholanic acid does not depress the melting point of 3( $\alpha$ )-hydroxy-12-keto

TABLE I

*Physical Constants of Samples of 3( $\alpha$ )-Hydroxy-12-keto- $\Delta^9,^{11}$ -cholanic Acid and Ester Prepared with Selenium Dioxide in Chlorobenzene-Acetic Acid (4.1)*

Sample No	Hrs	HCl in acetic acid 0.244 N	M p	Weight*	$\Gamma$ , $\lambda = 240$	Yield	Per cent of $\Delta^9,^{11}$	Per cent yield of $\Delta^9,^{11}$
		cc	°C	gm		per cent		
1A	24	0	173.0-173.5	32.60	8,950	84.0	76.0	63.9
1B			168.5-169.5	2.10	7,600	5.4	64.5	3.5
2A			173.5-174.5	25.00	9,350	64.4	79.5	51.2
2B	24	1.0	171.0-172.0	7.71	8,650	19.8	73.5	14.6
2C			169.0-170.5	1.87	7,840	4.8	66.6	3.2
3A	48	0	176.0-177.0	29.50	10,300	76.0	87.5	66.5
3B			176.0-177.0	4.50	10,230	11.6	87.0	10.1
4A	48	1.0	176.0-176.5	35.30	10,750	90.9	91.4	83.1
4B			173.0-174.0	0.50	9,050	1.3	77.0	1.0
5A	48	2.0	176.0-177.0	26.21	10,430	67.5	88.7	60.0
5B			175.0-176.0	6.70	9,700	17.3	82.1	14.3
5C			171.5-172.5	1.21	9,000	3.1	76.5	2.4
5D			168.0-169.5	0.95	8,070	2.4	68.5	1.6
6A	72	1.0	177.0-178.0	23.37	11,020	60.0	93.6	56.1
6B			176.0-177.0	8.38	10,780	21.6	91.5	19.7
6C			176.0-177.0	2.43	10,620	6.2	90.4	5.6
6D			161.0-168.0	0.79	9,940	2.0	84.5	1.7
6E			161.0-167.0	0.39	8,190	1.0	69.6	0.7
7†			180.0-180.5		11,770			
8‡			119.5-120.0		11,980			

\* 0.1 mole of methyl 3( $\alpha$ )-benzoxy-12-ketocholanate was used in each experiment. The theoretical yield of the unsaturated acid is 38.8 gm.

† Purified by crystallization of the sodium salt from normal sodium hydroxide.

‡ Ester prepared from Sample 7.

cholanic acid and that the melting point of a mixture of the methyl esters of these two acids is not depressed below that of the lower melting component. There is a relationship between the melting point and the extinction coefficient of mixtures of the two acids which is a rough indication of purity. A product with melting point of 175° contains approximately 80 per cent of the acid with double bond C<sub>9</sub>-C<sub>11</sub>, a sample with a melting point of 172° would contain approximately 72 per cent of the  $\Delta^9,^{11}$  derivative.

When the double bond is introduced at C<sub>7</sub>-C<sub>11</sub> by dehydrogenation with selenium dioxide, the product contains selenium, which must be removed

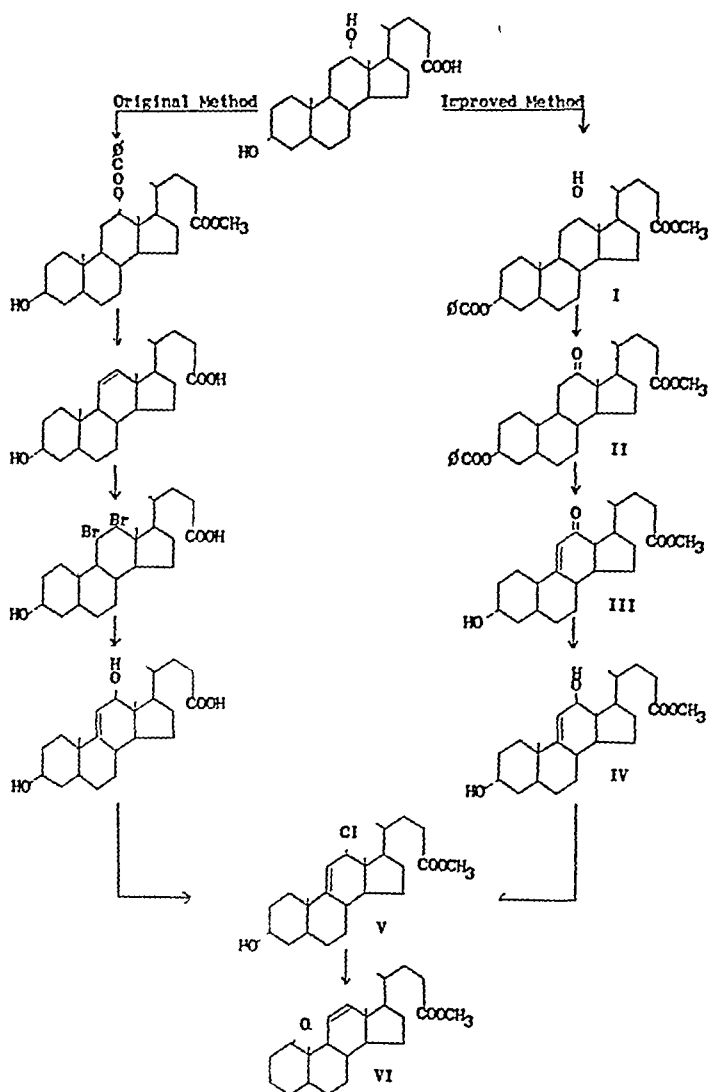


FIG 1 Original (left) and improved (right) methods of preparation of methyl 3,9 epoxy- $\Delta^{11}$  cholenate from desoxycholic acid

to permit reduction of the carbonyl group in methyl 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$   $\Delta^{11}$ -cholenate (III) with hydrogen and platinum. To devise a satisfactory method for the removal of selenium required prolonged investigation

After formation of the unsaturated keto compound with selenium dioxide, hydrolysis with alkali gave an aqueous solution of the sodium salt which was treated with zinc dust, Raney's alloy, copper citrate, hydrogen peroxide, or other reagents, but no one of these attempts was successful. In an acetic acid solution refluxing with silver or copper acetate or treatment with activated carbon failed to remove selenium. Purification by separation of the sodium salt, which is but slightly soluble in cold normal sodium hydroxide, is possible but the filtration is slow.

It was eventually found, however, that, after separation of the greater part of the selenium by filtration, treatment of the chlorobenzene acetic acid solution with a concentrated aqueous solution of chromic acid modified the selenium and decomposition products and resulted in removal of the selenium from the chlorobenzene phase.<sup>3</sup> The acetic acid, chromic acid, and chromium salts were washed out of the chlorobenzene with water, and after removal of the solvent the residue was hydrolyzed in methanol with alkali and the 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$  <sup>11</sup>-choleic acid was precipitated from aqueous solution with dilute acetic acid. The bulk of the acid was crystallized from cold 80 per cent acetone and the last portions from anhydrous acetone.<sup>4</sup>

Conversion of the 12-ketone of methyl 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$  <sup>11</sup>-choleate to a hydroxyl group can be accomplished by reduction with aluminum isopropoxide in isopropyl alcohol or with magnesium in methanol, but the most satisfactory method was found to be with hydrogen and Adams' platinum catalyst. In a solution of ethanol-acetic acid, 1:1, reduction of the 12-ketone to a hydroxyl group was almost quantitative. Determination of absorption in ultraviolet light indicated the presence of less than 2 per cent of the unsaturated ketone.<sup>5</sup>

A mixture of epimeric 12-hydroxy compounds was formed during the

<sup>3</sup> Chromic acid was used in the hope that small amounts of organic selenium compounds would be oxidized and that this would facilitate their removal. For this purpose chromic acid proved to be effective but in addition a strong influence was exerted on the physical state of the selenium which was present in colloidal form. The concentrated aqueous solution of chromic acid not only caused the separation of two phases but also brought about oxidation of a small amount of material which separated in insoluble form. These effects apparently supplied an active surface for the adsorption of the selenium. Only traces of selenium remained in the chlorobenzene phase.

<sup>4</sup> This unsaturated 12-keto acid has also been prepared from methyl 3( $\alpha$ )-acetoxy-12-ketocholinate by bromination at C<sub>11</sub> and subsequent dehydrobromination with either pyridine or collidine or with sodium ethylate, but the overall yields were lower than that obtained with selenium dioxide (5-7).

<sup>5</sup> The catalytic hydrogenation of methyl 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$  <sup>11</sup>-choleate will be discussed in Paper VIII. Formation of the 12-chloro and 12-methoxy compounds will be discussed in Paper IX.

reduction but treatment with 1 N hydrogen chloride in 84 per cent methanol and 16 per cent water gave only the 12-methoxy compound, already described (8), in excellent yield. This was converted into the 12-chloro derivative and thence into methyl 3,9-epoxy- $\Delta^{11}$ -cholelate (9)

TABLE II

*Yields of Intermediate Compounds in Preparation of Methyl 3,9-Epoxy  $\Delta^{11}$ -cholelate from Desoxycholic Acid*

Compound	Yield per cent
Methyl desoxycholate*	
" 3 benzoyldesoxycholate*	
" 3( $\alpha$ )-benzoxy 12 ketocholelate	90-95
3( $\alpha$ )-Hydroxy 12 keto $\Delta^9$ $^{11}$ cholemic acid	80-85
Methyl 3( $\alpha$ )-hydroxy-12 keto $\Delta^9$ $^{11}$ cholelate	95-98
" 3( $\alpha$ )-hydroxy-12 methoxy $\Delta^9$ $^{11}$ cholelate	88-92
" 3,9 epoxy- $\Delta^{11}$ cholelate	90-92

\* The yield of methyl desoxycholate and of the 3 benzoyl derivative depends on the quality of the commercial desoxycholic acid available. With highly purified material the yields are almost quantitative. With less pure desoxycholic acid, since the esterification is reversible, the mother liquors can be hydrolyzed and the recovered bile acid further purified and reesterified.

TABLE III

*Yields of Intermediate Compounds in Preparation of 3( $\alpha$ ) Hydroxy 11 ketocholelic Acid from Methyl 3,9 Epoxy- $\Delta^{11}$  cholelate*

Compound	Yield per cent
Methyl 3,9 epoxy-11,12 dibromocholelate, m p 143°*	75-77
" 3,9 epoxy-11 keto 12 bromocholelate	92-94
" 3,9 epoxy-11 ketocholelate	95-97
" 3( $\alpha$ ) hydroxy 11 keto 12 bromocholelate	87-89
" 3( $\alpha$ ) hydroxy 11 ketocholelate	90-93
3( $\alpha$ ) Hydroxy-11 ketocholelic acid	95-97

\* The yield of methyl 3,9 epoxy 11 12 dibromocholelate, m p 143°, is based on the theoretical yield from the starting material, methyl 3,9 epoxy  $\Delta^{11}$  cholelate. The yield of each intermediate is based on the theoretical weight from the preceding compound.

It is a matter of some interest to note the high yields of the several intermediate compounds which have been described. These are shown in Table II. The yield of methyl 3,9-epoxy- $\Delta^{11}$ -cholelate (VI) was about 60 per cent based on methyl desoxycholate. The yield of VI prepared by the

original method (Fig 1) through 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholenic acid and conversion of the 11,12-dibromide to 3( $\alpha$ ),12-dihydroxy- $\Delta^{9,11}$ -cholenic acid was less than 10 per cent. In addition, Table III shows the yields for the steps which include introduction of oxygen at C<sub>11</sub>.

#### EXPERIMENTAL

All melting points were determined on the Fisher-Johns apparatus.

*Desoxycholic Acid*—Treatment of crude desoxycholic acid with 6 volumes of benzene boiled under a reflux for 24 hours, followed by filtration and repetition for a second 24 hours, removed practically all fatty acids. This fraction varied from 3 to 8 per cent and consisted principally of palmitic acid. Digestion under a reflux with hot acetone-water, 4:1, for several hours in the proportion of 1 kilo of the bile acid to 6 liters of aqueous acetone dissolved all of the acid. The solution was cooled to 0° and filtered. About two-thirds of the starting material separated, which melted at 176–177°. Recrystallization from methyl ethyl ketone increased the melting point to 177–178° (10). Concentration of the mother liquor and recrystallization yielded more material with a melting point of 176–177°.

*Methyl Desoxycholate*—1200 gm of purified desoxycholic acid were dissolved in 3.6 liters of methanol and 40 cc of concentrated hydrochloric acid were added. After 3 hours at room temperature the solution was cooled to 5° and the following day the ester was removed by filtration. After addition of 40 gm of sodium bicarbonate the methanol was concentrated under reduced pressure and three more crops of crystals were obtained. The total weight was 1250 gm. Dane and Brady (11) have reported, and we have confirmed, that the ester separates from solution combined with 0.5 molecule of methanol of crystallization. The melting point of this ester with methanol of crystallization is not a satisfactory criterion of purity. Although the crystals partially melt at 82–83°, examination in polarized light reveals the presence of crystalline material even when the temperature is raised slowly to 100°.

*Methyl 3-Benzoyl desoxycholate*—1688 gm (4 moles) of methyl desoxycholate which contained 0.5 molecule of methanol were dissolved in 3400 cc of benzene which had been purified by treatment with concentrated sulfuric acid and distilled over sodium hydroxide. To remove methanol the benzene was evaporated under reduced pressure and the volume was made to 5200 cc with benzene. After addition of 400 cc of pyridine the solution was cooled to 20–25° and vigorously stirred. 467 cc of benzoyl chloride were added in 50 cc portions and the temperature was held between 20–25°. After 2 hours at room temperature the benzene solution was washed with water, with dilute hydrochloric acid, and again with water. After concentration of the benzene solution to a small volume the residue

was dissolved in 2 liters of methanol with the aid of heat. The product was separated 18 hours after the solution had been cooled to  $0^{\circ}$ . Concentration of the mother liquor gave successive crops with a total weight of 1820 gm.

1 gm. of methyl 3-benzoyldesoxycholate was dissolved in 35 cc. of dry ether. When the solution was concentrated, 0.880 gm. of the ester separated combined with 0.5 molecule of ether, m.p.  $93-94^{\circ}$ .

<i>Analysis</i> <sup>6</sup> — $C_{21}H_{34}O_5$	0.5( $C_6H_5$ )O	Calculated	C 74.75, H 9.40
		Found	" 74.35, " 9.40

The ether of crystallization is firmly held at room temperature. When dried at  $100^{\circ}$  and 0.1 mm., 5.5000 gm. of crystals lost 0.3760 gm. The loss calculated for 0.5 molecule of ether was 0.3720 gm.

<i>Analysis</i> <sup>7</sup> — $C_{21}H_{34}O_5$	Calculated, C 75.25, H 9.08, found, C 75.11, H 9.26
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Methyl 3-benzoyldesoxycholate separated from methanol with 0.5 molecule of methanol of crystallization, which was rapidly lost when dried at room temperature. When heated slowly, the methanol was gradually lost and the melting point was  $110-111^{\circ}$ , if heated rapidly, the melting point was  $83-84^{\circ}$  with effervescence.

*Methyl 3( $\alpha$ )-Benzoyl-12-ketocholanoate*—1986 gm. of methyl 3-benzoyldesoxycholate with methanol of crystallization were dissolved in 2200 cc. of chlorobenzene which was then removed under reduced pressure. 2200 cc. of chlorobenzene were added and again removed. The residue was dissolved in 7300 cc. of chlorobenzene and 1800 cc. of acetic acid. The solution was stirred mechanically and cooled to  $20-25^{\circ}$ . 366 gm. of chromic acid in 366 cc. of water were added in portions. After 1 hour 220 cc. of concentrated sulfuric acid were added and the solution was vigorously stirred for 1 hour at  $20-25^{\circ}$ . The organic phase was washed with 18 liters of water which contained 200 cc. of concentrated hydrochloric acid, since this was found to prevent formation of a persistent emulsion. After four washings with water the chlorobenzene solution was dried with sodium sulfate, filtered through infusorial earth, and concentrated to a small volume. The flask was warmed and 6 liters of methanol were added. The solution was cooled to  $5^{\circ}$  and allowed to stand for several hours. Three crops were separated 1679 gm., m.p.  $128.5-129^{\circ}$ , 162 gm., m.p.  $127-127.5^{\circ}$ , 9 gm., m.p.  $123-124^{\circ}$ .

For analysis<sup>7</sup> a sample was dried at  $110^{\circ}$  and 0.1 mm. for 4 hours.

$C_{32}H_{44}O_5$	Calculated, C 75.60, H 8.66, found, C 75.86, H 8.87
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<sup>6</sup> Analyses by Merck and Company, Inc., Rahway, New Jersey.

<sup>7</sup> Analysis by Dr. A. J. Haagen-Smit, William G. Kerckhoff Laboratories, California Institute of Technology, Pasadena, California.



*3(α)-Hydroxy-12-keto-Δ<sup>9,11</sup>-cholelic Acid*—508 gm of methyl 3(α)-benzoxy-12-ketocholanoate were dissolved in 3560 cc of chlorobenzene-acetic acid, 4 1 130 gm of selenium dioxide and 2 cc of 1 22 N hydrogen chloride in acetic acid were added and the solution was refluxed for 72 hours. Three solutions prepared as described were combined, the selenium was removed by filtration, 150 gm of chromic acid in 150 cc of water were added to the filtrate, and the solution was stirred vigorously for 2 hours. The chlorobenzene solution was decanted and again stirred for 2 hours with 75 gm of chromic acid in 75 cc of water. The organic phase was separated and washed four times with 8 liters of water. The chlorobenzene was removed under reduced pressure and the residue was dissolved in 4 liters of methanol which contained 400 cc of 18 N sodium hydroxide. The ester was hydrolyzed for 2 hours without the aid of heat, and water was added to dissolve the sodium salt which had separated. The solution was freed of methanol and the last traces of chlorobenzene under reduced pressure. The aqueous solution of the sodium salt was divided into two equal parts.

4 5 liters of water were heated to 100° in a 12 liter flask with a rapid stream of steam. 250 cc of acetic acid and a few crystals of 3(α)-hydroxy-12-keto-Δ<sup>9,11</sup>-cholelic acid were added and, while the solution was vigorously agitated with steam, one-half of the aqueous solution of the sodium salt was slowly added. The acid separated as a voluminous mass of needles and the suspension was maintained at 100° for 30 minutes. The second half was treated in the same manner. The flasks were cooled and the precipitate was filtered, washed, and dried to constant weight (1094 gm). The acid was then dissolved in acetone-water, 4 1. This step required refluxing for 1 to 2 hours with a volume of aqueous acetone equal to twice the weight of the crude acid. After cooling, 906 gm of acid were separated by filtration. A second crop of 149 gm and a third crop of 27 gm separated when the solution was concentrated.<sup>8</sup>

63 gm of 3(α)-hydroxy-12-keto-Δ<sup>9,11</sup>-cholelic acid, m p 178–179°, were dissolved in 500 cc of hot normal sodium hydroxide and 50 cc of methanol. The sodium salt was allowed to separate slowly at room temperature and the solution was held at 0° for 18 hours. Filtration removed a small amount of color and yielded the sodium salt, which was well washed with a sodium hydroxide at 5° and then dissolved in 500 cc of water and precipitated by addition to a hot dilute solution of acetic acid as described.

<sup>8</sup> No condition has been found for separation by crystallization of 3(α)-hydroxy-12-ketocholic acid from 3(α)-hydroxy-12-keto-Δ<sup>9,11</sup>-cholelic acid. For separation of the latter acid Hicks, Berg, and Wallis (5) have suggested the addition of gaseous hydrogen bromide to an ethereal solution, but, as will be shown elsewhere (Mattox, McKenzie, and Kendall, unpublished data), a hemihydrhalide is formed by both acids and no satisfactory separation is accomplished when both acids are present.

Crystallization from acetone-water, 4 l, gave a product which melted at 180–180.5° Log  $E = 4.070$  at  $\lambda = 240 \text{ m}\mu$   $[\alpha]_D = 107^\circ \pm 2^\circ$  ( $c = 1$  in methanol)

*Methyl 3( $\alpha$ )-Hydroxy-12-keto- $\Delta^9$   $^{11}$ -cholenate*—7.76 gm of 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$   $^{11}$ -cholenic acid, m p 180–180.5°, were esterified in 20 cc of methanol and 0.2 cc of concentrated aqueous hydrochloric acid. 10 cc of water containing 200 mg of sodium bicarbonate were added, the solution was cooled to 5°, and 7.36 gm of crystals melting at 117–119° were separated. The ester was recrystallized from a mixture of 22 cc of ether, 3 cc of methanol and 22 cc of petroleum ether. The melting point was 119.5–120° Log  $E = 4.077$  at  $\lambda = 240 \text{ m}\mu$   $[\alpha]_D = 107^\circ \pm 2^\circ$  ( $c = 1$  in methanol)

8.66 gm of 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$   $^{11}$ -cholenic acid, m p 179.5–180.5°, were esterified in methanol as described for the esterification of desoxycholic acid. The first crop weighed 8.04 gm and melted at 119.5–120°. Three more crops were obtained: 6.4 gm, m p 115.5–116.5°, 1.5 gm, m p 113.5–114.5°, 4 gm, m p 112.0–113.0°

*Methyl 3( $\alpha$ ) 12-Dihydroxy- $\Delta^9$   $^{11}$ -cholenate*—402 gm of methyl 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$   $^{11}$ -cholenate were dissolved in a mixture of 600 cc of acetic acid and 600 cc of 95 per cent ethanol and shaken in an atmosphere of hydrogen in the presence of 2 gm of Adams' platinum catalyst until the absorption of hydrogen had ceased. 94 hours were required and 1.00 mole of hydrogen was utilized.

A second 402 gm portion was reduced under the same conditions, except that a freshly prepared sample of platinum catalyst was used. In 63 hours 1.01 moles of hydrogen were absorbed.

The two solutions were combined, filtered from platinum, and evaporated under reduced pressure until a thick syrup remained. The syrup was dissolved in benzene and acetic acid was removed with water. The solution was concentrated and the benzene was displaced with methanol.

*Methyl 3( $\alpha$ )-Hydroxy-12-methoxy- $\Delta^9$   $^{11}$ -cholenate*—The syrupy residue described in the preceding paragraph was made to 3 liters with methanol and cooled to 5°. To the methanolic solution the following mixture was added: 390 cc of water, 334 cc of concentrated (37 per cent) hydrochloric acid, and sufficient methanol to make 1800 cc. After 40 days<sup>9</sup> the crystals which had separated were filtered from solution and washed with 600 cc of normal hydrochloric acid in 85 per cent methanol and with 8 liters of water. The weight was 757 gm (90.4 per cent) and the melting point was 160.5–162°. After crystallization from benzene the melting point was 162.5–163°. The

<sup>9</sup> Separation of the 12-methoxy compound takes place rapidly during the first 4 to 5 days. The last portions, however, require long treatment under the conditions which give the maximal yield.

preparation by other methods and analysis of this compound have been described (8, 9)

It is somewhat more convenient to perform the catalytic reduction in methanol and hydrochloric acid (0.05 N)<sup>10</sup> 402 gm of methyl 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$ - $\Delta^{11}$ -cholenate in 1200 cc of methanol were reduced with hydrogen in the presence of 2.00 gm of Adams' platinum oxide. 0.981 mole of hydrogen was absorbed in 12 hours. Without removal of the platinum the solution was made 84 per cent methanol, 16 per cent water, and 1 N hydrochloric acid. After 10 days 340 gm of the crude 12-methoxy compound were separated. After a total of 34 days 51 gm more were obtained.

The two portions of crystals were combined and recrystallized from 1200 cc of benzene. The benzene in the mother liquor was replaced with methanol and the solution was concentrated to small volume. The total weight of the 12-methoxy compound, with a melting point of 162–163° was 372 gm.

*Methyl 3,9-Epoxy- $\Delta^{11}$ -cholenate from Methyl 3( $\alpha$ )-Hydroxy-12-methoxy- $\Delta^9$ - $\Delta^{11}$ -cholenate*—A solution of 41.80 gm (0.1 mole) of methyl 3( $\alpha$ )-hydroxy-12-methoxy- $\Delta^9$ - $\Delta^{11}$ -cholenate in 200 cc of chloroform was vigorously stirred three times for 30 minutes each with 100 cc portions of concentrated hydrochloric acid and then with 500 cc of water. The intermediate compound, methyl 3( $\alpha$ )-hydroxy-12-chloro- $\Delta^9$ - $\Delta^{11}$ -cholenate, was not isolated. The chloroform solution was shaken vigorously in a mechanical shaker for 18 hours with a solution of 50 gm of sodium bicarbonate in 500 cc of water.

The chloroform solution was concentrated under reduced pressure and the chloroform was displaced with acetone. The volume was made to 100 cc with acetone, 50 cc of water were added, and the solution was cooled to 0°. The crystals which separated were filtered from solution and rinsed with cold 70 per cent acetone. The weight was 35.63 gm (92 per cent). The product melted at 53–54° and did not depress the melting point of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate.  $[\alpha]_D^{25} = -59^\circ \pm 2^\circ$  ( $c = 1$  in chloroform) (9).

#### SUMMARY

An improved method for the preparation of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate is described.

Methyl 3-benzoyldesoxycholate is oxidized to methyl 3( $\alpha$ )-benzoxy-12-ketocholenate and a double bond is introduced at C<sub>9</sub>–C<sub>11</sub> by dehydrogenation with selenium dioxide. Hydrolysis yields 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$ ,  $\Delta^{11}$ -cholonic acid. Esterification of the acid and reduction of the carbonyl group give methyl 3( $\alpha$ ), 12-dihydroxy- $\Delta^9$ - $\Delta^{11}$ -cholenate which is converted

<sup>10</sup> The use of methanolic HCl as a solvent for reduction was devised by Dr. Jacob van de Kamp in the Research Laboratories of Merck and Company, Inc.

through the 12-methoxy compound and the 12-chloro derivative into methyl 3,9-epoxy- $\Delta^{11}$ -cholenate. The over-all yield is about 60 per cent.

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## STEROIDS DERIVED FROM BILE ACIDS

### VII THE PROBABLE STEREOCHEMICAL CONFIGURATION OF SOME DERIVATIVES OF THE BILE ACIDS\*

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Derivatives of bile acids with substituents in Ring C have been described in the preceding papers of this series (1-6). It is the purpose of this paper to assign the probable stereochemical configurations for these compounds.

As desoxycholic acid (I)<sup>1</sup> is a convenient reference compound, it will first be necessary to discuss briefly the spatial arrangement of certain portions of this bile acid. The most probable configuration of desoxycholic acid is 3( $\alpha$ ), 12( $\alpha$ )-dihydroxycholan-ic acid (7-12).

Recent evidence for the configuration assigned to the hydroxyl group at C<sub>3</sub> has been furnished by the formation of 3,9-epoxy- $\Delta^{11}$ -cholenic acid (II) from methyl 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9$  <sup>11</sup>-cholenate (III) and its reconversion to III on treatment with hydrogen bromide (4). On the basis of these reactions the epoxide bridge of II must lie on the same side of the molecule as the 3-hydroxyl group of III.

Construction of models of these compounds with Stuart atoms (13) has shown that an oxygen bridge from C<sub>3</sub> to C<sub>9</sub> is essentially strain-free when in the  $\alpha$  position,<sup>2</sup> that is on the side opposite the angular methyl group at C<sub>10</sub>, provided that the A-B ring fusion is of the *cis*-decalin type. No epoxy models can be constructed with a *trans* A-B ring fusion, or in any case in which the oxygen atom occupies the  $\beta$  configuration. For these reasons the 3,9-epoxy compound appears to be 3( $\alpha$ ), 9( $\alpha$ )-epoxy- $\Delta^{11}$ -cholenic acid (II).<sup>3</sup>

Present chemical evidence indicates that the hydrogen at C<sub>9</sub> in the bile acids, cortical hormones, and so forth, has the  $\alpha$  configuration and the B-C ring fusion is *trans* (7, 15-17). Moreover, x-ray diffraction patterns are in agreement with this conclusion (8, 9, 18). Additional information on this

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<sup>1</sup> The roman numerals refer to the compounds in Table I.

<sup>2</sup> The terms  $\alpha$  and  $\beta$  are used in the sense which is discussed by Fieser (14).

<sup>3</sup> Since the epimeric 3( $\beta$ ), 9( $\beta$ )-epoxy compound appears to be excluded on steric grounds, in this paper the grouping will be designated 3,9 epoxy except when definite configurations are assigned.

point might be obtained by a further study of the physical properties of those compounds containing the 3,9-epoxy grouping, for in these substances the  $\alpha$  configuration at C<sub>9</sub> is established by the oxygen bridge. Thus, if the C<sub>9</sub> hydrogen of desoxycholic acid occupies the  $\alpha$  position, the configuration at C<sub>9</sub> of the 3,9-epoxide structure is the same as that of desoxycholic acid. On the other hand, if desoxycholic acid possesses hydrogen at C<sub>9</sub> in the  $\beta$  arrangement, the net result of conversion to the 3,9-epoxide would be an inversion of configuration with alteration in the B-C ring fusion from trans to cis. The deformation brought about by these changes can be readily demonstrated with models and should be susceptible of detection by physical methods. This correlation of configurations at C<sub>3</sub> and C<sub>9</sub> awaits further investigation.

The position of the hydroxyl group at C<sub>12</sub> has been the subject of considerable controversy. Caglioti and Giacomello, from the interpretation of x-ray diffraction patterns, suggested the  $\beta$  configuration (8, 18). On the other hand, while the early chemical results of Koechlin and Reichstein (11) were not conclusive, work in the laboratories of Gallagher and also of this institution indicates that the 12-hydroxyl group has the  $\alpha$  configuration (10, 19-21). More recent work of Sorokin and Reichstein confirms this conclusion (12). With the  $\alpha$  configuration assumed for the hydrogen at C<sub>9</sub> and for the hydroxyl groups at C<sub>3</sub> and C<sub>12</sub>, it is not difficult to assign the probable configurations for the compounds under discussion.

*Compounds Related to 3( $\alpha$ ),12( $\alpha$ )-Dihydroxycholan-ic Acid (Desoxycholic Acid)*—It has been shown that 3( $\alpha$ )-hydroxy-11,12-epoxycholan-ic acid (IV) made from 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholanic acid with perbenzoic acid can be reduced catalytically in acetic acid in the presence of a trace of hydrogen chloride to give desoxycholic acid (1). The oxide therefore has the  $\alpha$  configuration.<sup>4</sup> Gallagher and Long (10) have opened the 11,12-oxide to form methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-bromo-12( $\alpha$ )-hydroxycholanate (V). This configuration was indicated by the chemical properties of the bromoketone prepared by oxidation of the bromohydrin (V) with chromic acid. The compound, methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-bromo-12-ketocholanate (VI), thus obtained lost hydrogen bromide readily in boiling pyridine and yielded the  $\Delta^9$  compound. Seebeck and Reichstein (17) had previously shown that the same bromoketone prepared in another way was dehydrobrominated with pyridine and that 3( $\alpha$ )-acetoxy-11( $\alpha$ )-bromo-12-ketocholanate did not

<sup>4</sup> Alther and Reichstein reduced the 11,12-oxide (IV) with hydrogen in the presence of Ni and separated desoxycholic acid as the principal product (22). However, they accepted the interpretation of the x-ray diffraction patterns made by Caglioti and Giacomello (18) and assumed that the oxide and the hydroxyl group at C<sub>12</sub> have the  $\beta$  configuration. In a subsequent publication the oxide is assigned the  $\alpha$  configuration (23).

lose hydrogen bromide under the same conditions. The ease of dehydrobromination of VI is in keeping with the trans arrangement for the bromine at C<sub>11</sub> and the hydrogen at C<sub>9</sub>. The bromine at C<sub>11</sub> is therefore apparently in the  $\beta$  configuration.

Bromination of 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -choleic acid yields but a single dibromo derivative (VII), which can be separated in crystalline form (2). The configuration of the 2 atoms of bromine in VII is indicated by the following reactions. In anhydrous boiling pyridine 2 molecules of hydrogen bromide were removed and, after treatment with diazomethane, methyl 3,9-epoxy- $\Delta^{11}$ -choleate was isolated from the solution.<sup>5</sup> This result indicates that the primary step was loss of hydrogen bromide to form 3( $\alpha$ )-hydroxy-12-bromo- $\Delta^9$   $\Delta^{11}$ -choleic acid which then was converted into 3,9-epoxy- $\Delta^{11}$ -choleic acid (II) (4).

Treatment of the 11,12-dibromide (VII) with aqueous pyridine<sup>5</sup> or a dilute solution of sodium hydroxide (3, 24) removed both atoms of bromine and formed 3( $\alpha$ ),12-dihydroxy- $\Delta^9$   $\Delta^{11}$ -choleic acid (VIII). Because of the ready loss of hydrogen bromide from VII to form the 3,9-epoxy structure (II) in anhydrous medium and the 3( $\alpha$ ),12-dihydroxy derivative (VIII) in aqueous solution, it seems probable that the hydrogen at C<sub>9</sub> and the bromine at C<sub>11</sub> are trans to each other. This conclusion and the assumption that the atoms of bromine have added trans to each other are the basis for assigning 3( $\alpha$ )-hydroxy-11( $\beta$ ),12( $\alpha$ )-dibromocholeic acid as the probable configuration for VII.

*Compounds Related to 3( $\alpha$ ),12-Dihydroxy- $\Delta^9$   $\Delta^{11}$ -choleic Acid*—Gallagher and Long (10) showed that treatment of the 11( $\beta$ )-bromo-12( $\alpha$ )-hydroxy compound (V) with silver acetate in acetic acid yielded a fraction which after hydrolysis was identical with 3( $\alpha$ ),12-dihydroxy- $\Delta^9$   $\Delta^{11}$ -choleic acid (VIII) previously prepared by treatment of 3( $\alpha$ )-hydroxy-11( $\beta$ ),12( $\alpha$ )-dibromocholeic acid (VII) with alkali (3, 24). Unless inversion of the hydroxyl group at C<sub>12</sub> of the bromohydrin occurred during treatment with silver acetate (and this does not seem probable), the compound may be designated as 3( $\alpha$ ),12( $\alpha$ )-dihydroxy- $\Delta^9$   $\Delta^{11}$ -choleic acid (VIII).

With acetic anhydride and pyridine the methyl ester of the last mentioned compound forms a 3,12-diacetate (IX) which can be hydrolyzed with alkali to give the starting material. With acetic acid and a trace of sulfuric acid the 3( $\alpha$ ),12( $\alpha$ )-dihydroxy compound (VIII) very rapidly forms a monoacetyl derivative and from the rate of reaction and the conditions of the experiment it is evident that the formation of this acetate (X) is not by esterification but by replacement of the 12-hydroxyl group. Hydrolysis of the monoacetyl derivative restores the 3( $\alpha$ ),12( $\alpha$ )-dihydroxy compound (VIII). These results indicate that in the replacement of the 12-hydroxyl

<sup>5</sup> See the section "Results."



TABLE I

*Melting Points and Specific Rotations of Compounds Considered in This Paper*

Compound	M p	$[\alpha]_D$	Bibliographic reference No
	$^{\circ}\text{C}$	degrees	
I 3( $\alpha$ ),12( $\alpha$ )-Dihydroxycholan <sup>ic</sup> acid	177	+48	33, 34
II 3( $\alpha$ ),9( $\alpha$ )-Epoxy- $\Delta^{11}$ -chol <sup>enic</sup> "	158	-57	4
III Methyl 3( $\alpha$ )-hydroxy-12( $\alpha$ )-bromo- $\Delta^9$ <sup>11</sup> -chol <sup>enate</sup>	137	+213	4
IV 3( $\alpha$ )-Hydroxy-11( $\alpha$ ),12( $\alpha$ )-epoxycho- lan <sup>ic</sup> acid	167	+39	1, 10, 30
V Methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-bromo- 12( $\alpha$ )-hydroxycholan <sup>ate</sup>	139	+54	10
VI Methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-bromo-12- ketocho <sup>lanate</sup>	164	+41	10, 17
VII 3( $\alpha$ )-Hydroxy-11( $\beta$ ),12( $\alpha$ )-dibromocho- lan <sup>ic</sup> acid	179	+57	2
VIII 3( $\alpha$ ),12( $\alpha$ )-Dihydroxy- $\Delta^9$ , <sup>11</sup> -chol <sup>enic</sup> acid	200	+104	3, 10, 21
IX Methyl 3( $\alpha$ ),12( $\alpha$ )-diacetoxy- $\Delta^9$ <sup>11</sup> - chol <sup>enate</sup>	85	+198	3, 21
X 3( $\alpha$ )-Hydroxy-12( $\alpha$ )-acetoxy- $\Delta^9$ <sup>11</sup> - chol <sup>enic</sup> ac <sup>d</sup>	186	+206	3
XI 3( $\alpha$ ),12( $\beta$ )-Dihydroxy- $\Delta^9$ <sup>11</sup> chol <sup>enic</sup> acid	107*	+28	†
XII Methyl 3( $\alpha$ ),12( $\beta$ )-diacetoxy- $\Delta^9$ , <sup>11</sup> - chol <sup>enate</sup>	107	+3	†
XIII Methyl 3( $\alpha$ )-hydroxy-12( $\alpha$ )-methoxy- $\Delta^9$ <sup>11</sup> chol <sup>enate</sup>	163	+130	3
XIV Methyl 3 keto-12( $\alpha$ )-hydroxy- $\Delta^9$ <sup>11</sup> - chol <sup>enate</sup>	162	+70	3
XV Methyl 3 keto-12( $\alpha$ )-methoxy- $\Delta^9$ <sup>11</sup> - chol <sup>enate</sup>	100	+112	3
XVI Methyl 3-keto-12( $\alpha$ ) bromo- $\Delta^9$ <sup>11</sup> -cho- lan <sup>ate</sup>	137	+182	4
XVII Methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-hydroxy- 12( $\alpha$ )-bromocho <sup>lanate</sup>	203	+71	25
XVIII Methyl 3( $\alpha$ )-acetoxy-11-keto-12( $\alpha$ )- bromocho <sup>lanate</sup>	185	+8	5, 25
XIX Methyl 3( $\alpha$ ) acetoxy-11( $\beta$ ),12( $\beta$ )-epoxy cho <sup>lanate</sup>	155	+62	25
XX Methyl 3( $\alpha$ )-acetoxy-11( $\beta$ ) hydroxy- cho <sup>lanate</sup>	148	+69	5, 25, 27
XXI 3( $\alpha$ ) 11( $\beta$ )-Dihydroxycholan <sup>ic</sup> acid	201	+55	5
XXII 3( $\alpha$ ),11( $\alpha$ )-Dihydroxycholan <sup>ic</sup> "	147	+22	16
XXIII Methyl 3( $\alpha$ ),9( $\alpha$ ) epoxy-11( $\beta$ ),12( $\alpha$ )- dibromocho <sup>lanate</sup>	143	+45	4
XXIV Methyl 3( $\alpha$ ),9( $\alpha$ )-epoxy-11( $\alpha$ ),12( $\beta$ )- dibromocho <sup>lanate</sup>	123	+20	4

TABLE I—Concluded

Compound	M p	$[\alpha]_D$	Bibliographic reference No
	$^{\circ}\text{C}$	degrees	
XXV Methyl 3( $\alpha$ ),9( $\alpha$ )-epoxy-11( $\beta$ ) hydroxy-12( $\alpha$ )-bromocholananate	187	+53	5
XXVI Methyl 3( $\alpha$ ),9( $\alpha$ )-epoxy-11 keto 12( $\alpha$ )-bromocholananate	115	-36	5
XXVII Methyl 3( $\alpha$ ),9( $\alpha$ ) epoxy 11( $\beta$ ) acetoxymethyl 12( $\alpha$ )-bromocholananate	134	+17	4
XXVIII Methyl 3( $\alpha$ ),9( $\alpha$ ),11( $\beta$ ),12( $\beta$ )-diepoxycholananate	81	+16	4
XXIX Methyl 3( $\alpha$ ),9( $\alpha$ )-epoxy-11( $\beta$ )-acetoxymethyl 12( $\alpha$ )-bromocholananate	98	+50	This paper
XXX 3( $\alpha$ ),9( $\alpha$ )-Epoxy 11( $\beta$ )-hydroxycholanic acid	200	+56	" "
XXXI Methyl 3( $\alpha$ ),9( $\alpha$ ),11( $\alpha$ ),12( $\alpha$ )-diepoxycholananate	94	+16	4

\* Compound XI separated with 0.5 molecule of benzene and melted with effervescence. The specific rotation was taken on material which had been dried and freed of solvent of crystallization.

† Kendall, E. C., unpublished data.

group by the acetoxymethyl group inversion did not occur either with acetic anhydride in pyridine or with acetic acid and sulfuric acid, and that the 12-acetoxymethyl group in both IX and X has the  $\alpha$  configuration (3).

The epimeric 12-hydroxy compound (XI), to which we assign the  $\beta$  configuration at  $C_{12}$ , was prepared by catalytic reduction of 3( $\alpha$ )-hydroxy-12-keto- $\Delta^9$  11-cholenic acid.<sup>6</sup> When the methyl ester of XI was treated with acetic anhydride and pyridine, there was formed the 3( $\alpha$ ),12( $\beta$ )-diacetate (XII), which differed from IX and which with alkali yielded the dihydroxy starting material (XI). It is apparent that inversion did not occur either during acetylation or hydrolysis. However, when the 12( $\beta$ )-hydroxy compound (XI) was converted into the 12-acetate by treatment with a trace of sulfuric acid in acetic acid, the product isolated was identical with the 12( $\alpha$ )-acetate (X) formed from the 3( $\alpha$ ),12( $\alpha$ )-dihydroxy compound.<sup>6</sup>

This behavior of the 12( $\beta$ )-hydroxymethyl group in the presence of a trace of mineral acid indicates a strong tendency for a  $\beta$  substituent at  $C_{12}$  to invert to the  $\alpha$  position. Further evidence is afforded by treatment of the methyl esters of 12( $\alpha$ ) and 12( $\beta$ ) compounds (VIII and XI) with methanol and a trace of mineral acid. The same 12-methoxy compound was obtained from both epimeric forms. Moreover, the same 12-bromo derivative was formed from both epimeric hydroxy compounds by replacement with hydrogen

<sup>6</sup> Kendall *et al*, unpublished data.

biomide, and to the methoxy and bromo compounds (XIII and III), respectively, we assign the  $\alpha$  configuration at  $C_{12}$  (3, 4) <sup>6</sup> It has not been possible to accomplish a conversion of any compound with the 12( $\alpha$ ) configuration into one with a 12( $\beta$ ) arrangement by a replacement reaction

Regardless of the configuration at  $C_{12}$  of the starting material, the entering substituent becomes attached to the  $\alpha$  side of the molecule It would appear highly probable that steric hindrance is an important directive influence and we suggest that the entering group approaches the molecule on the side which is least hindered, that is, the side opposite the methyl group at  $C_{13}$

Compounds which have a ketone at  $C_3$ , methyl 3-keto-12-hydroxy- $\Delta^9$ , <sup>11</sup>-cholenate (XIV) and the closely related 12-methoxy (XV) or bromo (XVI) compounds, have the  $\alpha$  configuration at  $C_{12}$ , since they were prepared directly from 12( $\alpha$ ) derivatives or by methods analogous to those used to give the 12( $\alpha$ ) series with hydroxyl group at  $C_3$  (3, 4)

A second type of evidence for the configuration at  $C_{12}$  in the  $\Delta^9$ , <sup>11</sup> compounds is furnished by the difference in the reactivity of derivatives which are epimeric at  $C_{12}$  Hydrogenolysis of the hydroxyl group occurs with both forms but the rate is much faster with the compound to which the  $\alpha$  structure has been assigned than with the compound to which the  $\beta$  structure has been assigned This difference is exaggerated in the 3( $\alpha$ ), 12-diacetyl derivatives The 12( $\alpha$ )-acetoxyl group was readily removed by hydrogenolysis but the epimeric 12( $\beta$ ) compound remained unchanged under similar conditions <sup>6</sup> Presumably the differences of reactivity may be explained by the configuration at  $C_{12}$

The rates of formation of methyl 3( $\alpha$ )-hydroxy-12( $\alpha$ )-methoxy- $\Delta^9$ , <sup>11</sup>-cholenate in methanol with a trace of hydrogen chloride and of the corresponding 12( $\alpha$ )-acetoxyl compound in acetic acid with a trace of sulfuric acid are much faster with 3( $\alpha$ ), 12( $\alpha$ ) dihydroxy- $\Delta^9$ , <sup>11</sup>-cholenic acid than with the corresponding 12( $\beta$ )-hydroxy compound <sup>6</sup> These observations are in keeping with those already mentioned in regard to the reactivity of the 12( $\alpha$ ) and 12( $\beta$ ) compounds toward hydrogenolysis and they illustrate the influence of the stereochemical configuration on chemical reactivity

Still further evidence for the configuration of the substituent at  $C_{12}$  is furnished by the specific rotation There is but little difference in the specific rotations of some derivatives of 3( $\alpha$ )-hydroxycholenic acid which are epimeric at  $C_{12}$  but in the corresponding derivatives with a double bond  $C_9$ - $C_{11}$  the difference in specific rotations between the 12( $\alpha$ ) and 12( $\beta$ ) forms is marked Not only do those compounds to which the  $\alpha$  configuration has been assigned have rotations which are relatively high, but in each epimeric pair the rotation of the  $\alpha$  compound is higher than that of the compound with opposite configuration The specific rotations of the 12( $\alpha$ ) and 12( $\beta$ )

forms of 3( $\alpha$ ),12-dihydroxy- $\Delta^9$  <sup>11</sup>-cholenic acid are +104° and +28° respectively. The values for the 3( $\alpha$ )-acetoxy-12-hydroxy- $\Delta^9$  <sup>11</sup>-cholenic compounds are +116° and +46° and for the diacetates +200° and +3° (3).<sup>6</sup> Although it would be impossible to assign the configuration on the basis of specific rotation alone, in the three epimeric pairs cited the rotations are consistent with the configurations assigned.

*Compounds Related to 3( $\alpha$ ),11-Dihydroxycholanic Acid*—Ott and Reichstein (25) prepared methyl 3( $\alpha$ )-acetoxy-11-hydroxy-12-bromocholanate (XVII) and showed that this bromohydrin with chromic acid gave the 11-keto-12-bromo compound (XVIII). With alkaline aluminum oxide the bromohydrin (XVII) was converted into an 11,12-oxide (XIX).<sup>7</sup> Since the epimeric oxide (IV) (1, 10, 20) has been shown to have the  $\alpha$  configuration, the oxide (XIX) appears to have the  $\beta$  structure and this interpretation leads to methyl 3( $\alpha$ )-acetoxy-11( $\beta$ )-hydroxy-12( $\alpha$ )-bromocholanate as the most probable configuration for the bromohydrin (XVII) and methyl 3( $\alpha$ )-acetoxy-11-keto-12( $\alpha$ )-bromocholanate for the corresponding bromoketone (XVIII).

Ott and Reichstein (25) showed that catalytic reduction of the oxide (XIX) gave methyl 3( $\alpha$ )-acetoxy-11-hydroxycholanate (XX), to which they assigned the  $\alpha$  configuration at C<sub>11</sub>. Since the oxide (XIX) has now been shown to have the  $\beta$  configuration, it appears that the 11-hydroxyl group of methyl 3( $\alpha$ )-acetoxy-11-hydroxycholanate (XX) must also belong to the  $\beta$  series.<sup>8</sup>

Reduction of methyl 3( $\alpha$ )-acetoxy-11-ketocholanate gave the same 11( $\beta$ )-hydroxy product (XX) (27). This compound (XX) has also been prepared by reduction of 3( $\alpha$ )-hydroxy-11-ketocholanic acid to 3( $\alpha$ ),11( $\beta$ )-dihydroxycholanic acid (XXI) followed by esterification and acetylation (5).

Additional evidence in support of the 11( $\beta$ ) configuration in XXI follows from the resistance of the 11-hydroxyl group toward acetylation (5) and the ease with which the compound undergoes dehydration (27). The epimeric 11( $\alpha$ ) compound (XXII), prepared by Long and Gallagher (16), was readily acetylated and relatively resistant to dehydration of the 11-hydroxyl group.

*Compounds Related to 3,9-Epoxy- $\Delta^{11}$ -cholenic Acid*—It has been mentioned that bromination of 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholenic acid yields only a single dibromo compound (VII) which can be separated in crystalline form (2). However, bromination of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate results in the formation of two crystalline dibromo derivatives (XXIII and XXIV).

<sup>7</sup> Ott and Reichstein (25) assigned the  $\alpha$  configuration to the oxide (XIX) because the epimeric oxide (IV) had been designated as a member of the  $\beta$  series. In a subsequent publication the oxide (XIX) is assigned the  $\beta$  configuration (23).

<sup>8</sup> In a subsequent publication the hydroxyl group is assigned the  $\beta$  configuration (26).

(4) These dibromo compounds appear to be stereoisomers, since they are both converted with zinc into methyl 3,9-epoxy- $\Delta^{11}$ -cholenate (4)

It has not been possible to replace 1 of the atoms of bromine of XXIV selectively. However, the treatment of the dibromide (XXIII) with silver oxide in aqueous acetone gave methyl 3,9-epoxy-11-hydroxy-12-bromocholenate (XXV), which was oxidized to a bromoketone (XXVI). With hydrogen bromide the 3,9-epoxy structure was opened and from the solution methyl 3( $\alpha$ )-acetoxy-11-keto-12( $\alpha$ )-bromocholenate (XVIII) was separated in good yield. Debromination of XVIII and XXVI yielded methyl 3( $\alpha$ )-acetoxy-11-ketocholenate and methyl 3,9-epoxy-11-ketocholenate respectively. In the bromination of either of these two keto compounds it would be expected that both of the possible epimeric 12-bromo compounds would be formed, but in each instance only one bromo compound, XVIII or XXVI respectively, could be isolated. Thus it is evident that the configuration of the steroid nucleus exerted a strong directional influence.<sup>9</sup> On the basis of these results (5) we assign the  $\alpha$  configuration to the bromine at C<sub>12</sub> in methyl 3,9-epoxy-11,12-dibromocholenate (XXIII) and in methyl 3,9-epoxy-11-keto-12-bromocholenate (XXVI).

The bromohydrin (XXV) can be acetylated to give the bromoacetate (XXVII), also obtainable by treatment of methyl 3,9-epoxy-11,12-dibromocholenate (XXIII) with sodium acetate (4). Both the bromohydrin and its acetate yield an 11,12-oxide (XXVIII) with methanolic sodium hydroxide. Three mechanisms can be advanced for the conversion of the dibromide (XXIII) into the oxide (XXVIII). (1) The atoms of bromine in methyl 3,9-epoxy-11,12-dibromocholenate are *cis* with respect to each other. Replacement at C<sub>11</sub> is accompanied by inversion and the oxide is obtained by elimination of hydrogen bromide from a *trans* bromohydrin. (2) The epoxy dibromide is a *trans* dibromide, which undergoes replacement with inversion and yields a *cis* bromohydrin convertible to an oxide on treatment with alkali. (3) The epoxy dibromide is a *trans* dibromide and replacement at C<sub>11</sub> proceeds without inversion to a *trans* bromohydrin. This in turn gives the oxide with alkali.

Of these views the third is most acceptable. Addition of bromine to an olefinic linkage usually gives a *trans* product (28) and Bartlett (29) and Bartlett and White (30) have shown that oxides are readily formed from *trans* halohydrins and alkali, whereas the *cis* isomers are converted to ketones under similar conditions.

The work of Winstein and Buckles (31) indicates that when an atom of bromine is replaced by a substituent a second atom of bromine, if adjacent,

<sup>9</sup> Seebeck and Reichstein (17) found that in the bromination of 3( $\alpha$ )-acetoxy-12-ketocholenic acid the 11( $\alpha$ )-bromo derivative usually predominated. Gallagher and Long (20) have pointed out that in this reaction it could be anticipated that the hindered isomer at C<sub>11</sub> would be formed in less amount.

may participate in the replacement in such a manner that no inversion occurs. It therefore seems safe to conclude that the dibromide (XXIII) is methyl 3,9-epoxy-11( $\beta$ ),12( $\alpha$ )-dibromocholanate and the dibromide (XXIV) is methyl 3,9-epoxy-11( $\alpha$ ),12( $\beta$ )-dibromocholanate. The bromohydrin (XXV) is methyl 3,9-epoxy-11( $\beta$ )-hydroxy-12( $\alpha$ )-bromocholanate, the acetyl derivative (XXVII) has the  $\beta$  configuration at C<sub>11</sub>, and the oxide (XXVIII) is methyl 3( $\alpha$ ),9( $\alpha$ ),11( $\beta$ ),12( $\beta$ )-diepoxycholanate.

Halogen at C<sub>12</sub> in the bromoacetate (XXVII) was removed by catalytic debromination to give methyl 3,9-epoxy-11( $\beta$ )-acetoxycholanate (XXIX) and alkaline hydrolysis of the 11-acetate yielded 3,9-epoxy-11-hydroxy-cholanic acid (XXX).<sup>5</sup> Hydrolysis of the acetate required drastic treatment and it was found that under mild conditions the 11-hydroxyl group was neither acetylated nor replaced by chlorine with phosphorus pentachloride. Although the epimeric 11-hydroxy compound is not available for comparison, the chemical properties of XXX are in agreement with those anticipated for an 11( $\beta$ )-hydroxy compound.

A diepoxide, epimeric at 11,12 with methyl 3( $\alpha$ ),9( $\alpha$ ),11( $\beta$ ),12( $\beta$ )-diepoxycholanate (XXVIII), has been formed by the action of perbenzoic acid on 3,9-epoxy- $\Delta^{11}$ -cholanic acid (II). By exclusion the configuration of this oxide (XXXI) is assigned methyl 3( $\alpha$ ),9( $\alpha$ ),11( $\alpha$ ),12( $\alpha$ )-diepoxycholanate (4). The  $\alpha$  position is also indicated by the fact that perbenzoic acid forms the  $\alpha$  oxide with the closely related compound 3( $\alpha$ )-hydroxy- $\Delta^{11}$ -cholanic acid (1).

### Results<sup>10</sup>

All melting points were taken on the Fisher-Johns apparatus.

*Methyl 3,9-Epoxy- $\Delta^{11}$ -cholenate from 3( $\alpha$ )-Hydroxy-11( $\beta$ ),12( $\alpha$ )-dibromocholanic Acid (VII)*—A solution of 536 mg. of 3( $\alpha$ )-hydroxy-11( $\beta$ ),12( $\alpha$ )-dibromocholanic acid in 50 cc. of dry pyridine was boiled under a reflux condenser for 30 minutes. Ether and chloroform were added and the organic phase was washed with water, dilute sulfuric acid, again with water, and was filtered through sodium sulfate. The solution was esterified with diazomethane in ether and adsorbed on a column of 12 gm. of aluminum oxide. The material, eluted with 4:1 petroleum ether-benzene (177 mg.), was crystallized from cold dilute methanol to give 78 mg. of product which melted at 51–52° and did not depress the melting point of methyl 3,9-epoxy- $\Delta^{11}$ -cholenate [ $\alpha$ ]<sub>D</sub> =  $-59^\circ \pm 2^\circ$  (30.3 mg. in 3.00 cc. of chloroform).

*3( $\alpha$ ),12( $\alpha$ )-Dihydroxy- $\Delta^9$  <sup>11</sup>-cholanic Acid (VIII) from 3( $\alpha$ )-Hydroxy-*

<sup>10</sup> Some of the compounds described in this paper were analyzed in the laboratory of Merck and Company, Inc., Rahway, New Jersey, the remainder was analyzed by Mr. William Saschek in the Department of Biochemistry of Columbia University, New York.

*11(β),12(α)-dibromocholanic Acid (VII)*—A solution of 536 mg of 3(α)-hydroxy-11(β),12(α)-dibromocholanic acid (VII) in 50 cc of pyridine and 50 cc of water was boiled under a reflux condenser for 30 minutes. A mixture of ether and chloroform was added and the organic phase was washed with water, dilute sulfuric acid, and water and filtered through sodium sulfate. The halogen removed was 96 per cent of the theoretical amount. The solution was concentrated under reduced pressure and the residue was crystallized from benzene. The material, recrystallized twice from acetone, weighed 97 mg and when placed on the melting point stage at 190° melted at 193–195°. The melting point was not depressed when a sample of VIII was mixed with 3(α),12(α)-dihydroxy-Δ<sup>9,11</sup>-cholanic acid  $[\alpha]_D = +103^\circ \pm 2^\circ$  (31.4 mg in 3.00 cc of methanol).

*Methyl 3,9-Epoxy-11(β)-acetoxycholananate (XXIX) from Methyl 3,9-Epoxy-11(β)-acetox-12(α)-bromocholananate (XXVII)*—A solution of 525 mg of methyl 3,9-epoxy-11(β)-acetox-12(α)-bromocholananate in 2 cc of pyridine and 100 cc of ethanol was shaken in an atmosphere of hydrogen with 5.0 gm of palladium catalyst (32) for 6.5 hours. The solution was filtered and the filtrate was concentrated under reduced pressure. The residue was dissolved in benzene and the solution was washed with dilute acetic acid and with water and concentrated to dryness. The residue crystallized from dilute methanol to give 240 mg of product which melted at 95–96°. Further purification from the same solvent raised the melting point to 97.5–98°.  $[\alpha]_D = +50^\circ \pm 2^\circ$  (20.0 mg in 4.00 cc of chloroform).

$C_{27}H_{42}O_6$  Calculated, C 72.61, H 9.48, found, C 72.59, H 9.58

3,9-Epoxy-11(β)-acetoxycholanic acid was obtained by hydrolysis of 446 mg of methyl 3,9-epoxy-11(β)-acetoxycholananate (XXIX) in 50 cc of methanol and 25 cc of 1 N aqueous sodium hydroxide. The solution was refluxed for 2 hours, acidified with acetic acid, and concentrated under reduced pressure. The residue was extracted with benzene and the solution was washed with water and concentrated to dryness under reduced pressure. After three crystallizations from ligroin the material melted at 163–163.5°.  $[\alpha]_D = +54^\circ \pm 1^\circ$  (40.0 mg in 4.00 cc of chloroform).

$C_{26}H_{40}O_5$  Calculated, C 72.19, H 9.32, found, C 71.93, H 9.18

*3,9-Epoxy-11(β)-hydroxycholanic Acid (XXX) from 3,9-Epoxy-11(β)-acetoxycholanic Acid*—92 mg of 3,9-epoxy-11(β)-acetoxycholanic acid were dissolved in 5 cc of methanol, 7.5 cc of 5 N aqueous sodium hydroxide were added, and the solution was refluxed for 16 hours. The methanol was removed in an air current, water was added, and the solution was acidified with hydrochloric acid. The resulting precipitate was collected, washed, and crystallized from dilute acetone. The product (70 mg) melted at 199–200°.  $[\alpha]_D = +56^\circ \pm 2^\circ$  (24.6 mg in 3.00 cc of chloroform).

$C_{21}H_{35}O_4$  Calculated, C 73.80, H 9.81, found, C 73.98, H 9.72

3,9-Epoxy-11( $\beta$ )-hydroxycholan-ic acid has also been prepared through hydrolysis of the 11( $\beta$ )-formate. Treatment of the dibromide (XXIII) in methanol with potassium formate gave a mixture of the 11( $\beta$ )-formoxy-12( $\alpha$ )-bromo and the 11( $\beta$ )-hydroxy-12( $\alpha$ )-bromo compounds. Catalytic debromination of either the bromoformate or the bromohydrin, followed by alkaline hydrolysis, yielded the 11( $\beta$ )-hydroxy acid (XXX).

*3,9-Epoxy-11-ketocholan-ic Acid from 3,9-Epoxy-11( $\beta$ )-hydroxycholan-ic Acid (XXX)*—780 mg of 3,9-epoxy-11( $\beta$ )-hydroxycholan-ic acid were oxidized at room temperature in 10 cc of chloroform and 30 cc of acetic acid with 5.40 cc of 1.85 N chromic acid dissolved in 95 per cent acetic acid. After 16 hours water and benzene were added and the organic phase was washed with water and evaporated to dryness under reduced pressure. After two recrystallizations from dilute acetone the residue gave 454 mg of material which melted at 173–174°. Some samples of this compound partially melted at about 168°, resolidified, and melted at 173–174°. The melting point was not depressed when the crystals were mixed with an authentic sample of 3,9-epoxy-11-ketocholan-ic acid (5)  $[\alpha]_D^{20} = +92^\circ \pm 2^\circ$  (20.0 mg in 4.00 cc of chloroform).

$C_{21}H_{33}O_4$  Calculated, C 74.18, H 9.33, found, C 73.94, H 9.53

#### SUMMARY

The probable stereochemical configurations are assigned for twenty-five derivatives of the bile acids with one or more substituents in Ring C. These compounds are related to cholan-ic acid,  $\Delta^9$ -<sup>11</sup>-cholen-ic acid, and  $\Delta^{11}$ -cholen-ic acid and, in addition, position 3 is substituted with a ketone or an  $\alpha$ -hydroxyl group, or is attached to position 9 through an epoxide.

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# THE INFLUENCE OF THYROID ACTIVITY ON THE LIVER AND PLASMA LIPIDES OF CHOLINE- AND CYSTINE-DEFICIENT RATS

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The effects of choline deficiency on liver lipides have been well established and the variations in plasma lipides encountered in clinical hypo- and hyperthyroid states have been thoroughly studied. With the recent finding that hypothyroid animals do not exhibit the hepatic damage which results from chronic choline deficiency in normal rats (1)<sup>1</sup> it was thought of interest to determine the influence of thyroid activity on the plasma and liver lipides of both choline- and cystine-deficient animals.

## EXPERIMENTAL

The subjects of this study were male rats of the Vanderbilt strain (2). After weaning they were grown to 100 gm. on a stock ration and then housed in individual cages and fed the experimental diets. The basal diet for all groups was prepared as follows: casein 10, sucrose 59, cotton seed oil 10, lard 15, cod liver oil 2, salts (3) 3, cholesterol 0.5, and inositol 0.3. In addition, to each kilo of diet were added thiamine hydrochloride 3 mg., riboflavin 5 mg., pyridoxine 3 mg., calcium pantothenate 50 mg., niacin 500 mg., 2-methyl-1,4-naphthoquinone 3 mg.,  $\alpha$ -tocopherol 50 mg., and *p*-aminobenzoic acid 100 mg. All rats received 5  $\gamma$  each of both folic acid and biotin twice weekly by pipette. The following materials were incorporated in the various diets, as specified in Tables I and II, at the expense of an equivalent amount of sucrose: choline chloride 0.6, cystine 0.5, thiouracil 0.3, desiccated whole thyroid powder (Armour) 0.2, 0.3, and 0.4 per cent. Hyperthyroidism was produced by feeding thyroid powder and hypothyroidism both by thiouracil feeding and by total thyroidectomy performed under ether anesthesia when the rats weighed 80 gm. A number of thyroidectomized animals are not included in Tables I and II, as they never properly recovered from the surgical procedure and failed to eat or grow adequately.

At the conclusion of the experiments the animals were mildly anesthetized with nembutal given intraperitoneally, the jugular vein was exposed by proper dissection, and 0.1 ml. of heparin solution (Lederle) was administered intravenously. About 1 minute later, the animals were exsanguinated by

<sup>1</sup> Handler, P., and Follis, R. H., Jr., in preparation.

bilateral carotid section and the blood collected in a small porcelain dish. By this procedure one may consistently obtain 6 to 8 ml of blood from a 150 gm rat and proportionately more from larger animals. The blood was immediately centrifuged and the plasma saved for analysis. The rats' livers were removed, weighed, and transferred to a bottle containing an alcohol-ether (3:1) mixture.

The analytical procedures were similar to those previously employed but with some modification. The livers were transferred to the container of a Waring blender and ground with 8 ml of alcohol-ether for each gm of liver. The suspension was transferred to a beaker and refluxed for 5 minutes. A round bottom flask with running water was used as a crude but efficient condenser. The suspension was filtered, the insoluble matter washed with ether, and the combined filtrates evaporated. The residue was extracted with warm petroleum ether, and the solution filtered through anhydrous sodium sulfate, evaporated, and weighed. This value was taken as the total lipide content of the liver. In a batch of control livers from normal stock rats, the results were virtually identical with values obtained by prolonged extraction of dried livers with chloroform in a Soxhlet apparatus. For further analysis the lipides were dissolved in petroleum ether and suitable aliquots removed. Total cholesterol was determined colorimetrically by the Liebermann-Burchard reaction with a Coleman spectrophotometer and lipide phosphorus determined by the Fiske-Subbaw procedure after perchloric acid digestion. Total lipides, cholesterol, and phospholipides were determined in the same manner on the alcohol-ether-soluble fraction of heparinized plasma. Phospholipides were calculated by assuming an average of 4 per cent as the phosphorus content of phospholipides.

The first series of animals was maintained on the experimental rations for 3 weeks. The nature of the various groups and the results obtained are summarized in Table I. The animals of Groups A to D may be taken as the controls, from the nutritional standpoint, for this series. Groups E to H were choline-deficient, Groups J to L were cystine-deficient, and Groups M to O deficient in both choline and cystine. The groups in each nutritional category are then arranged in order of ascending thyroid activity.

With respect to the nutritional variables, in animals with no altered thyroid function, the results were in accord with previous findings. Thus, simple choline-deficient rats showed markedly fatty livers and increased liver cholesterol concentration. Neither liver fat nor cholesterol increased quite so much in animals deficient in both cystine and choline. The livers of rats receiving choline but not cystine contained less total lipide and cholesterol than any of the other groups. It should be noted that the level of choline administration employed under these conditions was just sufficient to maintain normal liver lipide and cholesterol concentrations. This pro-

vided a rather labile situation, so that the effects of varying thyroid activity would be more readily apparent

The most dramatic effect of varying thyroid activity noted, under these conditions, was on the liver cholesterol fraction and is readily apparent in each nutritional category. Hypothyroidism invariably resulted in an elevated liver cholesterol concentration and hyperthyroidism in a marked decrease therein. In consequence, the highest cholesterol concentration in the series was observed in thyroidectomized, choline-deficient rats, while

TABLE I

*Effects of Thyroid Activity on Liver Lipides of Choline- and Cystine Deficient Rats*

Group	Dietary supplement	Level of thyroid activity	No of rats	Food intake gm per day	Weight gain gm	Liver			
						Weight	Total lipides	Cholesterol	Phospholipides
						gm	per cent wet weight	per cent wet weight	per cent wet weight
A	Choline + cystine	Thyroidectomy	8	9.8	35	6.10	9.4	1.10	2.31
B	" + "	Thiouracil	8	8.8	30	7.43	11.0	1.03	2.53
C	" + "	Normal	16	10.7	44	7.05	7.1	0.35	2.52
D	" + "	Thyroid fed*	8	9.9	33	5.55	4.6	0.19	2.54
E	Cystine	Thyroidectomy	8	9.7	31	8.37	24.3	1.61	1.63
F	"	Thiouracil	8	8.6	27	9.70	30.5	1.23	1.54
G	"	Normal	16	10.3	36	10.11	23.5	0.77	1.68
H	"	Thyroid fed*	8	10.4	28	8.43	17.8	0.39	1.89
J	Choline	Thiouracil	8	6.5	23	5.27	5.4	0.39	2.46
K	"	Normal	16	7.1	20	4.93	5.3	0.29	2.57
L	"	Thyroid fed*	8	7.5	24	5.38	4.1	0.16	2.51
M	None	Thiouracil	8	6.9	26	6.61	21.3	0.78	1.76
N	"	Normal	16	6.8	22	6.79	16.1	0.41	2.04
O	"	Thyroid fed*	8	7.4	17	6.54	9.7	0.26	2.31

\* 0.3 per cent in diet

the lowest was in the thyroid-fed, cystine-deficient group. The magnitude of this difference is, perhaps, more impressive when the total amounts of liver cholesterol in each group are compared. These may be readily calculated from the data of Table I. Thus, while the cholesterol concentration in Group E was 10 times that of Group L, the total liver cholesterols were 134.0 mg and 8.6 mg, respectively, while food consumption was 9.7 and 7.5 gm per day respectively. The effects of varying the level of thyroid activity on the neutral fat fraction of the liver lipides were not quite as striking. In fact, in each nutritional category, comparison of hyper- or hypothyroid animals with the normal controls of that category does not reveal differences which are more than barely statistically significant.

However, when the hyperthyroid group is compared with the hypothyroid group, it becomes obvious that qualitatively the neutral fat behaves in a pattern similar to that described for cholesterol, *viz*, rising in the hypothyroid state and falling in the hyperthyroid state. In accord with previous findings (4, 5), the phospholipide fraction of liver under these circumstances is reduced in otherwise fatty livers. This fall, however, is deceptive, since it represents merely dilution by the other lipide components and actually the ratio of phospholipide protein N of the liver remains fairly constant. Neither the nutritional nor the thyroid variations produced any change in phospholipide concentration which was not accountable in these terms.

While the extent to which liver fat accumulates in choline-deficient rats is, in considerable measure, also a function of the growth rate (4, 6, 7), this variable does not seem to have materially affected the present results. It is patently impossible to obtain maximal growth under the dietary circumstances employed in the production of choline deficiency. Therefore, the animals of Group C must be taken as the "normal controls" in this series. When that is done, it can be seen that in this category the thyroid-fed and thyroidectomized rats grew at equal rates, about 75 per cent of that of the controls, yet the effects on liver lipides were entirely different. A similar situation was observed in the choline-deficient category. None of the rats in the two cystine-deficient categories grew well and the differences apparent in growth rate among the various groups are statistically insignificant. The one anomalous finding in the entire series was the greater neutral fat concentration in Group F than Group E. Considering the remainder of the series it appears doubtful that this had any real physiological significance.

In the second series, the experimental period was twice that of the first series, *viz*, 6 weeks. The nature of the various groups and the results are summarized in Table II. While not indicated in Table II, cystine was added to all the diets of this series and so, nutritionally, there are presented only control and choline-deficient animals. While Table II indicates that twelve rats were used in each group, actually the entire experiment was performed twice, each time with six rats per group. The two experiments were in excellent agreement and the values shown in Table II were calculated as if a single large study had been made.

In most respects the results obtained in this series were in agreement with those found in the shorter trial. Again, the most prominent deviation from the basal conditions induced by alteration of the level of thyroid activity was in the cholesterol fraction of the liver lipides. In both nutritional categories, the livers of thyroidectomized animals contained about twice as much cholesterol as those of the basal animals, while the livers of the hyperthyroid rats contained somewhat less than half as much cholesterol.

as those of the basal animals. Moreover, when compared with the values of Table I, it can be seen that cholesterol continued to accumulate in the livers of thyroidectomized rats in the second 3 weeks. As in the first series, the effects of altered thyroid function on the neutral fat fraction were comparatively small, albeit, nevertheless, real and similar in direction to those shown in Table I. The failure of thiouracil to yield results comparable with those of thyroidectomy may, perhaps, be attributed to the toxicity of this compound. Thus, the rats of Groups B and G ate less and grew more

TABLE II

*Effects of Thyroid Activity on Plasma and Liver Lipides of Choline-Deficient Rats*

Group	Dietary supplement	Thyroid activity	Food intake	Weight gain	Liver				Plasma		
					Weight	Total lipides	Cholesterol	Phospholipides	Total lipides	Cholesterol	Phospholipides
			gm per day	gm	gm	per cent	per cent	per cent	mg per 100 ml	mg per 100 ml	mg per 100 ml
A	None	Thyroidectomy	7.8	68	11.62	24.63	0.3	1.3	477	124	58
B	"	Thiouracil	6.5	33	7.30	15.31	1.20	1.8	500	130	60
C	"	Normal	10.6	88	13.41	18.21	1.51	1.7	303	86	53
D	"	Thyroid fed 0.2%	11.1	63	10.41	17.30	0.81	1.9	307	89	55
E	"	" " 0.4%	10.5	42	9.27	13.80	0.53	1.4	290	81	37
F	Choline	Thyroidectomy	8.3	73	8.10	10.11	1.54	2.2	695	165	75
G	"	Thiouracil	6.3	37	6.90	10.41	1.50	2.1	728	171	82
H	"	Normal	9.8	95	9.07	8.00	0.80	2.4	410	101	60
J	"	Thyroid fed 0.2%	11.3	54	8.04	7.10	0.41	2.5	341	100	68
K	"	" " 0.4%	10.1	31	8.02	6.20	0.36	2.0	335	93	58

\* Each group consisted of twelve rats

slowly than did the basal controls or thyroidectomized rats. In the same manner, while the rats of Groups E and K ate more than those of Groups C and H for 5 weeks, their appetites dwindled in the last week, accompanied by a weight loss of about 4 gm, and it is difficult to determine the extent to which this influenced the results. Nevertheless, the over-all pattern seems clearly established. In evaluating these data it must be realized that the basal diet for this study contained 0.5 per cent cholesterol, which undoubtedly would exaggerate any physiological circumstance which would tend to permit an accumulation of hepatic cholesterol, although essentially normal values were obtained in the control rats of both series.

While choline deficiency and hypothyroidism exert similar influences on liver lipides, their effects on plasma lipides are opposite in direction. Choline deficiency resulted in a small, but real, decrease in all fractions of the

plasma lipides, while hypothyroidism, in both nutritional categories, resulted in a marked rise in the plasma lipides which was somewhat more striking in the choline-fed than in the choline-deficient rats. Hyperthyroidism occasioned a comparatively slight decrease in the plasma lipides. These findings, in direction, are all in keeping with clinical experience with alterations in human thyroid function. In the main, total lipides, cholesterol, and phospholipides behaved in a parallel manner, rising and falling under the same circumstances and in a roughly proportional fashion. Of these, the phospholipide fraction was perhaps the least consistent. It should be remarked that the values for plasma phospholipide found in these rats were considerably lower than those usually seen in human subjects. The mean value for plasma lipid phosphorus concentration for the entire series was 2.4 mg per 100 ml with a range between 1.5 and 3.3. Normal human plasma contains 8 to 11 mg of lipid phosphorus per 100 ml with considerably higher values found in the hyperlipemic plasma of hypothyroid individuals. Similar low values for lipid phosphorus in rat plasma have recently been observed by others.<sup>2</sup>

#### DISCUSSION

From the data presented in Tables I and II it would seem established that the concentration of cholesterol in the livers of both normal and choline-deficient rats varies inversely with the level of thyroid activity. These observations are in accord with the findings of Forbes (8) that thyroxine administration augmented the lipotropic action of choline at moderate levels of choline feeding. It is unfortunate that this author's data did not permit an evaluation of the effects of thyroxine in choline-deficient rats. In another paper from the same laboratory (9) it was found that thiourea feeding was without effect on liver fat or cholesterol concentration. In view of the present findings this may, perhaps, indicate merely that thiourea is not as effective as thiouracil or thyroidectomy in the production of an experimental hypothyroid state. No definitive statement concerning the influence of thyroid activity on the neutral fat of the liver can be made. According to the present data hyperthyroidism depresses the accumulation of neutral fat in the livers of both control and choline-deficient animals, while hypothyroid function operates in opposite fashion. However, the differences were small and, while statistically significant, there was some overlapping of the various groups. Since the extent of neutral fat accumulation in choline deficiency is, in a large measure, dependent also upon such factors as food consumption, growth rate, toxic agents, and the supply of other dietary essentials (1, 6), these relatively small differences found at

<sup>2</sup> Artom, C, personal communication

various levels of thyroid activity are difficult to evaluate. Nevertheless, comparison of the lipide content of hyperthyroid and hypothyroid livers indicates that the behavior of neutral fat parallels that of cholesterol under these circumstances.

In the present study, the effects of cystine feeding were equally pronounced on the cholesterol and neutral fat fractions of the liver lipides. This was readily apparent at all levels of thyroid activity. Since it has been stated that the increased liver fat content which results from feeding cystine to choline-deficient rats is the result of an augmented rate of fatty acid synthesis (10), this would suggest that cystine feeding may accelerate the production of some common precursor of both fatty acids and cholesterol, perhaps at the 2-carbon stage.

The present data do not permit a categorical statement concerning the mechanism by which the thyroid regulates fat metabolism. Hypothyroidism results in a relative hyperlipemia and an increased liver fat content, while hyperthyroidism has the opposite effect. Since the influence of the thyroid is most marked on the liver cholesterol fraction, it seems possible that the thyroid specifically controls cholesterol metabolism. That the increase in the neutral fat of liver and plasma and of plasma phospholipides which results from hypothyroidism may all be secondary to the behavior of cholesterol under these conditions appears quite likely in view of the work of Popjak (11), who has shown that feeding emulsified free cholesterol results in a marked rise in the concentration of all plasma lipides at the expense of depot neutral fat. Since no measurements of extrahepatic tissue fat were made in the present study, it is not possible to state whether the thyroid regulates the rate of cholesterol synthesis and utilization or its transport and distribution. This question can best be answered with the aid of the isotope tracer technique.

Hypothyroidism induced by thyroidectomy<sup>1</sup> or by feeding thiouracil (1), *p*-aminobenzoic acid,<sup>1</sup> or sulfonamides<sup>1</sup> protects rats against the cirrhosis which usually results from the ingestion of choline-deficient diets. In the present study it has been found that, with all other conditions being held constant, hypothyroid activity actually augments the usual accumulation of liver lipides in choline-deficient rats. It appears, therefore, that unless hepatic metabolism is proceeding at the rate dictated by at least normal thyroid function, the severe hepatic necrosis and fibrosis of choline deficiency do not occur, despite the fact that the parenchymatous liver cells are engorged with masses of lipide material. This situation is analogous to the previous finding that the extremely fatty livers which result from the addition of nicotinamide to a diet containing 18 per cent casein do not undergo the usual necrosis and fibrosis observed in the fatty livers resulting from choline deficiency on a low protein diet (7). It recalls also the fact



that liver damage due to experimental hyperthyroidism has only been observed when accompanied by some other toxic agent such as carbon tetrachloride (12), anoxia (13), staphylococcus toxin (14), or rabbit papilloma virus (15), although none of these are, of themselves, hepatotoxic under the conditions employed

The alterations in the levels of plasma lipides which were induced by choline deficiency and by varying levels of thyroid activity were in accord with all previous findings. The diminution in plasma lipides is consistent with the concept that the fatty liver of choline-deficient rats is due to a failure of phospholipide synthesis and consequently of fat transport from the liver. Hyperthyroidism did not diminish the plasma lipides as markedly as is sometimes encountered in Grave's disease. Hypothyroidism, however, resulted in an elevation of plasma lipides which was more pronounced in choline-fed than in choline-deficient animals. Experimentally, the hyperlipemia of hypothyroidism has been observed in dogs (16) and rabbits (17), but not monkeys (18). In the dog, the degree of the lipemia was determined by the animal's appetite. It may well be that the failure to find an appreciable drop in the plasma lipide concentration of thyroid-fed rats and the exaggerated hyperlipemic response to thyroidectomy and thiouracil feeding was the result of the use of a basal diet high in fat and cholesterol in the present study.

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#### SUMMARY

1 Hypothyroidism, produced both by thyroidectomy and by thiouracil feeding, resulted in a marked increase in the cholesterol concentration and a relatively small increase in the neutral fat content of the livers of both control and choline-deficient rats.

2 Thyroid feeding resulted in a pronounced decrease in the cholesterol concentration and a slight decrease in the neutral fat concentration of the livers of both control and choline-deficient rats.

3 Cystine deficiency partially prevented the accumulation of neutral fat and cholesterol in the livers of normal and choline-deficient rats. The effects of cystine deficiency and thyroid feeding were cumulative and cystine deficiency partially offset the accumulation of liver lipides in hypothyroid rats.

4 The plasma lipide concentrations of choline-deficient rats were some-

what reduced below those of normal rats Thyroid feeding slightly diminished plasma lipides of both series, while in hypothyroidism the plasma lipide concentrations of both series were greatly increased

5 The significance of these findings is discussed

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# HISTOCHEMICAL DISTRIBUTION OF PEPTIDASE ACTIVITY IN THE CENTRAL NERVOUS SYSTEM OF THE RAT

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Recent advances in knowledge of the action of tissue proteolytic enzymes have indicated that they may play a role in both normal and abnormal growth processes (1-3). As a measure of one aspect of proteolytic activity and as a background for a survey of the distribution and types of specificity of catheptic enzymes in the central nervous system and the neoplasms to which its cellular constituents give rise, the peptidase activity of different portions of the nervous system has been investigated.

Previous studies on proteolytic enzymes in nervous tissue (4-6) have been upon extracts of whole brain or peripheral nerve and have demonstrated the presence in such preparations of enzymes capable of splitting a number of protein and peptide substrates. In addition, Zeller and Maritz (7) have reported that characteristic patterns of proteolytic activity are exhibited by different portions of the forebrains of several species. Because of the variations in cellular composition of different portions of the central nervous system, it seemed desirable to compare the proteolytic activity of each of its principal divisions against a single substrate, and to use micro-analytical methods in order to obtain simultaneous histological control of the observations made.

## Methods

Adult rats of Wistar strain were killed by decapitation, and the brain and upper dorsal and cervical portions of the spinal cord removed immediately and frozen in a mixture of crushed dry ice in petroleum ether (temperature  $-78^{\circ}$ ). For purposes of comparative enzyme estimation, the brain was divided into forebrain (cerebral hemispheres), brain stem, and cerebellum by section of the appropriate peduncles, and portions of the cervical segments of spinal cord were similarly studied. The technique for preparation of the tissue was that developed by Linderstrøm-Lang and Mogensen (8), and for quantitative histochemical determination of peptidase activity the method of Linderstrøm-Lang and Holter (9) was employed without essential changes. Uniform cylinders, 4 mm in diameter, of the tissues were punched out (in the case of the spinal cord, coronal segments were used), and serial frozen sections, 20  $\mu$  in thickness,

cut from each with a microtome maintained at  $-15^{\circ}$  in a cryostat. For each observation, four uniform serial sections were prepared.

The first section was fixed in 10 per cent formalin and stained by the Giemsa method for microscopic examination.

The second and third sections were extracted for 20 to 24 hours at  $5^{\circ}$  with 10.3 c mm of 30 per cent glycerol solution buffered with 4 per cent  $m/15$  phosphate at pH 7.0. The extracts, including the sections, were then used respectively as the experimental and autolysis control preparations for determination of the amount of cleavage of 6.9 c mm of a 0.2  $M$  solution of *DL*-alanylglycine in 0.0343  $N$  NaOH after suitable periods of incubation of enzyme extract with substrate at  $38^{\circ}$ . The racemic dipeptide was used as in the original method. Activators of proteolytic enzymes, *e.g.* cysteine and manganese, were not included in the reaction mixture. It has been shown that the splitting of *DL*-alanylglycine by yeast and intestinal mucosa is not enhanced by such substances and may even be partially inhibited (10, 11). Furthermore, it seemed reasonable to obtain comparative data under essentially *in vivo* environmental conditions. The pH was, therefore, maintained at 7.0, and effective buffering found to take place, since the reaction of the mixture varied inappreciably during the course of hydrolysis. Liberated amino groups were determined by titration in acetone with 0.05  $N$  HCl in 95 per cent alcohol with naphthyl red as indicator, the proteolytic reactions having previously been terminated by addition of 33.8 c mm of 0.05  $N$  HCl in acetone to the enzyme-substrate mixture.

The fourth section of each series was placed in a desiccator over phosphoric anhydride and left overnight in a cryostat at  $0^{\circ}$  to  $-15^{\circ}$ . Its dry weight was subsequently determined by means of the quartz torsion balance described by Lowry (12). Some of these sections were then extracted in xylene for 5 to 10 minutes, and reweighed to obtain their fat-free dry weights.

In most instances, duplicate determinations were made for each experimental point, but some single observations are included in the data presented. Peptidase activity has been expressed in terms of the volume of 0.05  $N$  HCl necessary to neutralize the freed amino groups per microgram of dry weight of the adjacent section of the series.

#### RESULTS AND DISCUSSION

Preliminary experiments were carried out to establish the relationship between the length of the period of hydrolysis and the amount of splitting of equivalent amounts of substrate by extracts of sections of four principal divisions of the central nervous system: the cerebral hemispheres, brain stem, cerebellum, and spinal cord. Typical examples of the peptidase activity of each as a function of length of hydrolysis are presented graphi-

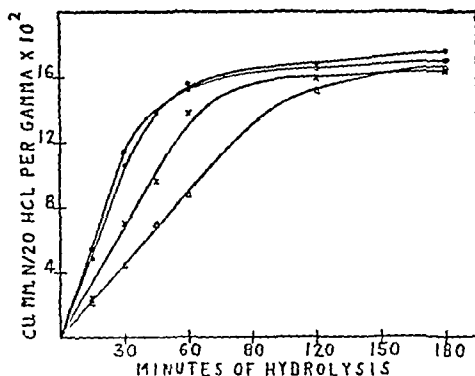


FIG 1 Relation of peptidase activity of various portions of the central nervous system to the length of enzymatic hydrolysis (● cerebrum, ○ cerebellum, × brain stem, △ spinal cord)

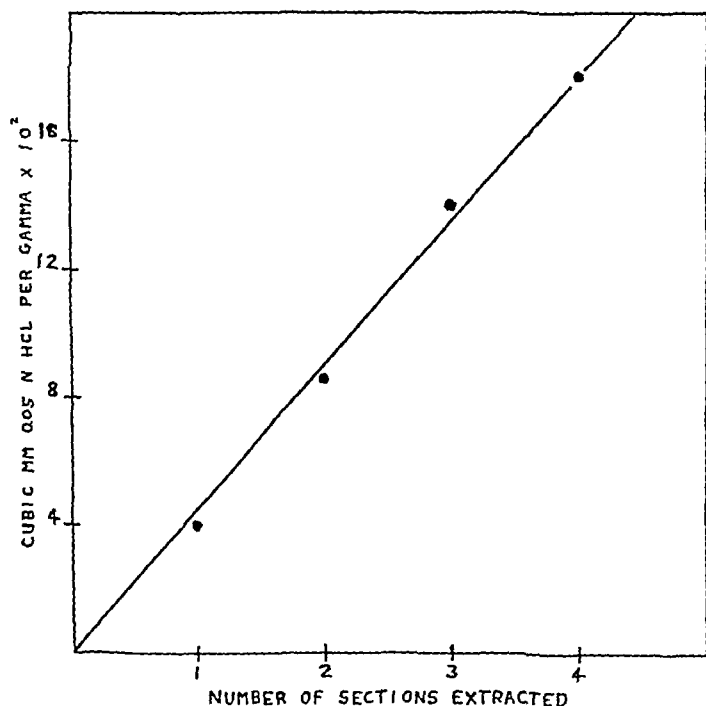


FIG 2 Relation of peptidase activity of glycerol extracts to the number of uniformly cut microtome sections of cerebral hemisphere extracted

cally in Fig 1. The enzymatic splitting of the substrate is clearly a linear function of the hydrolytic period for about 45 minutes, and, therefore, within this temporal range the kinetics of the reaction are of zero order

TABLE I

*Comparative Peptidase Activity of Cerebral Hemisphere, Brain Stem, Cerebellum, and Spinal Cord*

Portion of central nervous system	Experiment No	Titer of 0.05 N HCl	Dry weight of section	Titer of HCl per $\gamma$ dry weight	Average titer of duplicate $\times 10$	Fat free dry weight	Titer of HCl per $\gamma$ fat free weight	Average titer per $\gamma$ fat free weight $\times 10$
		<i>c mm</i>	$\gamma$	<i>c mm</i>	<i>c mm</i>	$\gamma$	<i>c mm</i>	<i>c mm</i>
Cerebral hemisphere	1	5.35	38.1	0.140	14.0	30.3	0.178	17.8
	2	5.63	46.0	0.122	12.2	37.5	0.150	15.0
	3	6.41	52.3	0.122	12.7	41.8	0.153	16.5
		6.25	47.3	0.132		35.4	0.177	
	4	4.90	56.7	0.086	8.8	37.1	0.132	13.2
		5.25	58.4	0.090				
	5	6.04	45.3	0.133	12.9	37.8	0.160	16.0
		5.97	47.7	0.125				
	6	6.10	53.8	0.113	11.4	39.1	0.156	15.6
		5.14	44.8	0.115		33.1	0.155	
	7	5.89	58.8	0.100	10.0	45.3	0.130	13.0
	8	6.20	58.4	0.106	10.6	39.0	0.159	15.9
Average S.E.	9	5.41	49.2	0.110	11.0	34.1	0.159	15.9
	12	8.52	71.0	0.120	12.0	51.3	0.166	16.6
					11.6 $\pm 0.46$			15.6 $\pm 0.43$
Brain stem	4	4.15	62.4	0.067	6.7			
		4.29	65.0	0.066				
	5	5.05	66.8	0.076	8.1	41.0	0.123	12.6
		4.27	50.3	0.085		33.1	0.129	
	8	4.85	64.9	0.075	7.5	38.3	0.127	12.7
	9	5.08	70.1	0.073	7.3	46.3	0.110	11.0
	10	5.47	80.4	0.068	7.4	53.0	0.103	11.2
		5.88	73.5	0.080		48.5	0.121	
	12	7.00	64.0	0.109	10.9	47.4	0.148	14.8
	13	4.45	59.2	0.075	7.8	46.3	0.096	10.1
						41.9	0.105	
					8.0 $\pm 0.49$			12.1 $\pm 0.61$
Cerebellum	4	6.11	61.9	0.099	10.1	40.0	0.153	15.3
		6.52	63.2	0.103				
	5	6.93	49.8	0.139	13.0	37.8	0.183	17.0
		6.77	56.4	0.120		43.4	0.156	
	6	6.52	55.6	0.117	11.2	38.9	0.168	16.5
		6.20	57.8	0.107		38.2	0.162	
	8	6.37	64.8	0.098	9.8	41.6	0.153	15.3
	9	7.16	67.4	0.106	11.1	46.2	0.155	17.0

TABLE I—Concluded

TABLE I—Concluded

Portion of central nervous system	Experiment No	Titer of 0.05% HCl	Dry weight of section	Titer of HCl per g dry weight	Average titer of duplicate $\times 10^2$	Fat free dry weight	Titer of HCl per g fat free weight	Average titer per g fat free weight $\times 10^2$	
		<i>c mm</i>	<i>g</i>	<i>c mm</i>	<i>c mm</i>	<i>g</i>	<i>c mm</i>	<i>c mm</i>	
Cerebellum—continued	10	6 77	58 2	0 116	15 4	36 9	0 184	18 9	
		7 98	51 3	0 155		42 3	0 189		
		8 04	52 8	0 152		43 6	0 189		
		8 35	46 8	0 178		38 9	0 215		
		6 73	64 4	0 105		41 9	0 161		
	12	6 90	67 3	0 103	12 4 $\pm 0.96$	44 2	0 156	17 2 $\pm 0.71$	
	13								
Average									
S E									
Spinal cord	4	1 28	18 8	0 068	6 8	15 3	0 084	8 4	
	5	3 42	49 7	0 069	7 2	32 7	0 105	11 5	
		3 87	52 4	0 074	6 3	31 1	0 124	10 1	
		3 07	48 5	0 063		30 3	0 101		
	6	4 10	68 8	0 060	6 0	26 0	0 137	13 7	
	8	2 38	43 4	0 055	6 4				
	9	3 57	49 8	0 072	8 2	47 1	0 102	12 3	
		4 82	71 0	0 068		44 0	0 144		
		6 35	66 8	0 095		29 4	0 128		
	12	3 76	43 9	0 086	8 6			12 8	
Average					7 1 $\pm 0.34$			11 5 $\pm 0.74$	
S E									

Consequently, in order to insure comparability of results for the subsequent experiments on the comparative peptidase activity of different portions of the nervous system, a standard incubation period of 30 minutes was used, assuring that the cleavage of substrate would be independent of the concentration of the latter and dependent only on the amount of the enzyme present

In order to determine whether the peptidase activity is directly related to the number of sections extracted, an experiment was performed in which duplicate sets of one, two, three, and four sections of cerebral hemisphere were extracted, and the activity of the extracts measured in the presence of the sections after 10 minutes of hydrolysis of the substrate by each. The results are plotted in Fig 2 which shows that the enzyme activity is a linear function of the number of sections extracted

Finally, that complete extraction of the enzyme was being brought about by the procedure was demonstrated by extracting single sections with one, two, three, and four aliquots of glycerol solution and finding that the re-



sulting extracts exhibited similar degrees of peptidase activity (averages of duplicate determinations following 30 minutes hydrolysis were 0.099, 0.085, 0.093, 0.093 c mm of 0.05 N HCl per  $\gamma$  of dry weight of section respectively)

A series of experiments was then carried out to compare the peptidase activity in extracts of sections cut from cerebral hemispheres, cerebellum, brain stem, and spinal cord. Table I summarizes the results of these experiments.

The data show that on a dry weight basis the cerebral and cerebellar hemispheres exhibited a significantly greater degree of peptidase activity than did the spinal cord and brain stem. The titer of 0.05 N HCl (c mm) necessary to neutralize the amino groups liberated during 30 minutes of hydrolysis per microgram of dry weight of section averaged 0.116 ( $\pm$  standard error 0.005) for cerebral hemisphere, 0.080 ( $\pm$  s.e. 0.005) for brain stem, 0.124 ( $\pm$  s.e. 0.010) for cerebellum, and 0.071 ( $\pm$  s.e. 0.003) for spinal cord.

The anatomical controls revealed an expectedly larger proportion of gray matter and hence of nerve cell bodies in the sections of cerebrum and cerebellum than in those of brain stem and spinal cord. Because of the higher proportion of medullated fibers in the brain stem and spinal cord, some of the desiccated sections from each group were defatted in xylene, and the enzyme activities expressed in terms of the fat-free dry weights (Table I). The results show that significant differences in activities between the groups also exist even when the myelin sheaths are ruled out as contributors to the mass of tissue. The average titers of HCl expressed in terms of xylene-defatted dry weights amounted to 0.156 c mm ( $\pm$  s.e. 0.004) for cerebral hemisphere, 0.121 ( $\pm$  s.e. 0.006) for brain stem, 0.172 ( $\pm$  s.e. 0.007) for cerebellum, and 0.115 ( $\pm$  s.e. 0.007) for spinal cord.

These results suggest that nerve cell bodies and then dendrites have a higher peptidase activity than their axones. To verify this impression, an experiment was performed on sections of the corpus callosum which contains no nerve cell bodies. The average titer of nine determinations was only 0.046 c mm of 0.05 N HCl per microgram of dry weight and 0.064 c mm per microgram of dry weight of xylene-defatted material.

Thus, it appears that the peptidase activity is more closely related to the number of neuronal cell bodies than to the total protoplasmic mass. Experiments are at present under way to test the validity of this hypothesis by measurement of the laminar distribution of peptidase activity in the cerebral cortex as a function of the density of the nerve cell body population. At any rate, it is apparent that determinations of proteolytic activity made on preparations of whole brain are not necessarily representative of any of its parts, and that such studies must take into account the

striking anatomical variations which different parts of the central nervous system display

#### SUMMARY

Quantitative histochemical estimations of peptidase activity in the central nervous system of the rat have revealed that the splitting of DL-alanylglycine is accomplished more rapidly by extracts of the cerebral and cerebellar hemispheres than by similar preparations of brain stem and spinal cord

The degree of enzyme activity appears to be related to the number of nerve cell bodies present in the sample of tissue analyzed

The authors gratefully acknowledge their indebtedness to Professor A B Hastings for invaluable assistance during pursuit of the foregoing studies

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# THE INHIBITION OF MICROBIAL GROWTH BY $\beta$ -2-FURYLALANINE\*

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The effect of  $\beta$ -2-thienyl-DL-alanine, an isostere of phenylalanine, upon the growth of *Saccharomyces cerevisiae*, *Escherichia coli*, and certain other microorganisms has been reported by du Vigneaud and coworkers (1, 2) and by Beerstecher and Shive (3). Thienylalanine was inhibitory toward the growth of these organisms, and the inhibition was counteracted by phenylalanine. These investigators found that as the concentration of phenylalanine in the medium was increased larger amounts of thienylalanine were required to produce inhibition of growth. Thus, thienylalanine was shown to act as an "antiphenylalanine" for these microorganisms.

Since furan compounds are isosteres of analogous thiophene compounds (4), it seemed desirable to study the effect of  $\beta$ -2-furylalanine on the growth of microorganisms and to compare its activity with that of  $\beta$ -2-thienylalanine.

In this paper the authors wish to report the inhibitory action of  $\beta$ -2-furyl-DL-alanine upon the growth of *Saccharomyces cerevisiae* and *Escherichia coli* and the effects of naturally occurring amino acids on the toxicity of furylalanine.

## EXPERIMENTAL

**$\beta$ -2-Furyl-DL-alanine**—The method described by Herz, Dittmer, and Cristol (5) was employed for the preparation of  $\beta$ -2-furyl-DL-alanine which was used in all of the experiments described below.

**Amino Acids**—Most of the amino acids used were obtained from commercial sources. L-Proline and hydroxy-L-proline were obtained through the courtesy of Dr. E. E. Howe of Merck and Company.

**Inhibition of Growth of *Saccharomyces cerevisiae*, Fleischmann's Strain 139**—The technique followed in the yeast growth experiments and the medium and supplement used in this work with yeast were the same as those previously described (2, 6) except that commercial sucrose was used without purification. 6.2 ml. of medium were added to 1.0 ml. of water containing

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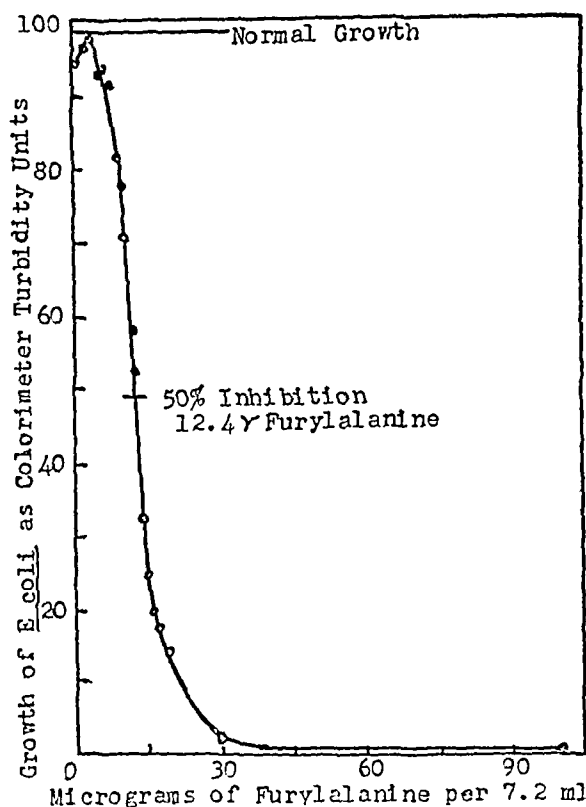
FIG 2 Inhibition of the growth of *Escherichia coli* by furylalanine

TABLE III

*Inhibition of Growth of Escherichia coli by Furylalanine in Presence of Phenylalanine*

DL-Phenylalanine added per 7.2 ml	Furyl DL alanine added per 7.2 ml for 50 per cent inhibition of growth		Furylalanine Phenylalanine
$\gamma$	$\gamma$	$\gamma^*$	molar ratio
0	13.2		
0.5	30.8	17.6	38
1.0	49.5	36.3	39
2.0	60.6	47.4	26
5.0	170	157	33
10	845	832	88
20	1770	1757	93

\* Values in this column are the amounts of furylalanine that had to be added above that amount required to produce 50 per cent inhibition without added phenylalanine

required to produce 50 per cent inhibition of growth. When inhibition was obtained in the presence of various amounts of DL-phenylalanine, additional furyl-DL-alanine was required to produce 50 per cent inhibition of growth. The ratio of additional furylalanine to phenylalanine present was not constant at the levels tested, as is shown in Table III.

With increasing amounts of furyl-DL-alanine, larger amounts of DL-phenylalanine were required to nullify by half the toxicity of the furylalanine, the ratio of these quantities was nearly constant at higher levels of furylalanine, as is shown in Table IV

TABLE IV  
Nullification by Phenylalanine of Inhibition of *Escherichia coli* Growth by Furylalanine

Furyl DL-alanine per 7.2 ml	DL Phenylalanine required for 50 per cent nullification of toxicity	$\frac{\text{Furylalanine}}{\text{Phenylalanine}}$
$\gamma$	$\gamma$	molar ratio
25	0.36	74
250	4.2	64
500	6.5	82
1000	12.7	82
1500	18.0	88
2500	31.8	84

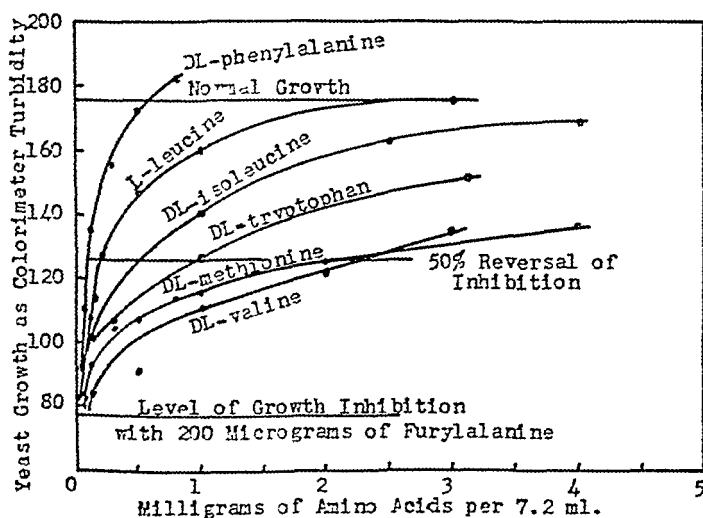


FIG 3 The effect of various amino acids on the toxicity of 200  $\gamma$  of furylalanine on the growth of *Saccharomyces cerevisiae*

*Effect of Amino Acids Other Than DL-Phenylalanine in Nullifying Furylalanine Toxicity*—A level of 200  $\gamma$  of furyl-DL-alanine per 7.2 ml was chosen as a reference point at which to study the effect of amino acids other than phenylalanine on the furylalanine toxicity on yeast. The growth curves in Fig 3 illustrate the results obtained with the amino acids which showed appreciable activity in counteracting the toxicity of furylalanine

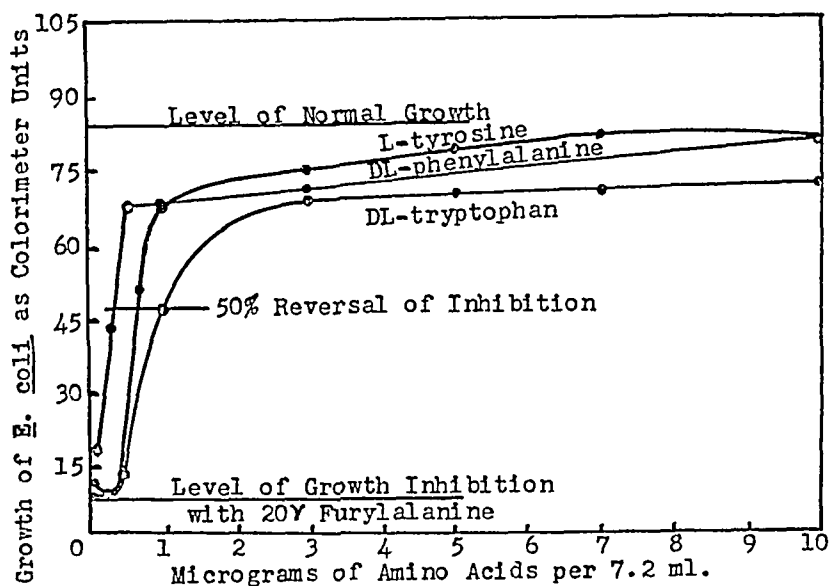


FIG 4 The effect of DL phenylalanine, L-tyrosine, and DL-tryptophan on the toxicity of 20  $\gamma$  of furylalanine on the growth of *Escherichia coli*

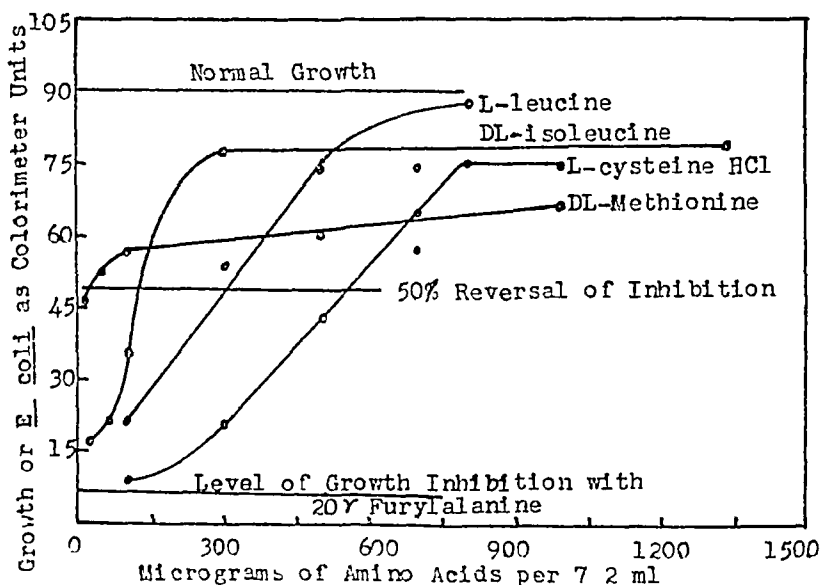


FIG 5 The effect of slightly active amino acids on the toxicity of 20  $\gamma$  of furylalanine on the growth of *Escherichia coli*

For a similar study with *Escherichia coli*, a level of 20  $\gamma$  of furylalanine per 7.2 ml was selected. The results obtained are illustrated by the curves in Figs 4 and 5.

The effects of most of the naturally occurring amino acids on the toxicity

of furylalanine on both *Saccharomyces cerevisiae* and *Escherichia coli* are shown in Table V

Those amino acids which counteracted the growth inhibition of either organism are listed first, with their relative effectiveness compared with DL-phenylalanine taken as 100 per cent. The comparisons were made at the levels of amino acids which were necessary to obtain growth at a level half way between normal growth and the level of inhibited growth due to

TABLE V

*Nullification of Furylalanine Inhibition of Growth of Saccharomyces cerevisiae and Escherichia coli by Various Amino Acids*

For <i>S. cerevisiae</i>			For <i>E. coli</i>		
Amino acid	Amount required for 50 per cent nullification of inhibition by 200 $\gamma$ furylalanine	Effectiveness on molar basis	Amino acid	Amount required for 50 per cent nullification of inhibition by 20 $\gamma$ furylalanine	Effectiveness on molar basis
	$\gamma$	per cent		$\gamma$	per cent
DL-Phenylalanine	90	100	DL-Phenylalanine	0.34	100
L-Leucine	200	36	L-Tyrosine	0.68	55
DL-Isoleucine	550	13	DL-Tryptophan	1.0	42
DL-Tryptophan	950	12	DL-Methionine	50	0.61
DL-Methionine	2300	3.5	DL-Isoleucine	68	0.40
DL-Valine	2200	2.9	L-Leucine	225	0.12
DL-Alanine	6000	<1.0	L-Cysteine HCl	420	0.06*
			L-Proline	1950	0.05*
			L-Histidine HCl - H <sub>2</sub> O	4280	0.05*
Inactive L-tyrosine, L-cysteine HCl, L-proline, L-histidine HCl			Inactive DL-valine and DL-alanine		

Inactive for both organisms: glycine, DL-serine, DL-threonine, L-aspartic acid, L-asparagine, L-glutamic acid, hydroxy-L-proline, L-lysine HCl, L-cystine

\* The possibility of contamination with more active amino acids is not ruled out for these results

the presence of 200  $\gamma$  of furylalanine in the case of yeast and 20  $\gamma$  with *Escherichia coli*. This is the growth level which represents 50 per cent nullification of the toxicity of furylalanine.

#### DISCUSSION

It will be noted from the data of Table V and Figs 3, 4, and 5 that phenylalanine is the most active amino acid for the reversal of the furylalanine inhibition of both yeast and *Escherichia coli* growth. This would



indicate that furylalanine, like thienylalanine, is an "antiphenylalanine" for these two organisms. However, leucine, isoleucine, and tryptophan also have an appreciable effect on inhibition of yeast growth by furylalanine. For the reversal of the inhibition of the growth of *E. coli*, tyrosine and tryptophan had a high degree of activity. This is similar to the effect that these amino acids have on the toxicity of thienylalanine. Beerstecher and Shive (3) suggested that tryptophan is a precursor of phenylalanine for the strain of *E. coli* which they used in their work. Just in what way the

TABLE VI

Comparison of Effects of  $\beta$ -2-Thienyl-DL-alanine and  $\beta$ -2-Furyl-DL-alanine on Growth of *Saccharomyces cerevisiae* and *Escherichia coli*

Properties compared	For <i>S. cerevisiae</i>		For <i>E. coli</i>	
	Thienylalanine	Furylalanine	Thienylalanine	Furylalanine
Amount required for 50% inhibition of growth	42.5 $\gamma$	140 $\gamma$	1.88 $\gamma$ per 6.0 ml	12.4 $\gamma$ per 7.2 ml
Ratio, antagonist to phenylalanine for inhibition in presence of phenylalanine	0.83	2.0	24*	Not constant
Ratio, antagonist to phenylalanine for reversal of inhibition	0.55	1.83	22*	84*

Amino acids reversing toxicity

	per cent	per cent	per cent	per cent
DL-Phenylalanine	100†	100†	100†	100†
L-Tyrosine	Inactive	Inactive	140	55
DL-Tryptophan	8.0	12.0	78	42
L-Leucine	13.2 (DL-)	36 (L-)	0.15 (DL-)	0.12 (L-)
DL-Isoleucine	9.8	13	0	0.4

\* Constant at higher levels only

† In this comparison, the activity of phenylalanine is taken as 100 per cent

amino acids which showed an appreciable activity in counteracting the "antiphenylalanine" properties of furylalanine are related to phenylalanine remains to be determined.

A comparison of the properties of thienylalanine (2) and those of furylalanine shows that these two metabolite antagonists are acting in identical manners, thus can be seen by the comparative data of Table VI, which indicate that  $\beta$ -2-furyl-DL-alanine is approximately 30 per cent as active toward *Saccharomyces cerevisiae* and 15 per cent as active toward *Escherichia coli* as  $\beta$ -2-thienyl-DL-alanine.

The authors wish to express their appreciation to Mrs Hester Purdy McNulty and Mrs Charmion McMillan for checking some of the microbiological results

#### SUMMARY

$\beta$ -2-Furyl-DL-alanine inhibited the growth of *Saccharomyces cerevisiae* and *Escherichia coli* and phenylalanine reversed this toxicity. To inhibit growth to 50 per cent of normal required 140  $\gamma$  and 12.4  $\gamma$  per 7.2 ml for yeast and *Escherichia coli*, respectively.

For *Saccharomyces cerevisiae*, L-leucine, DL-tryptophan, and DL-isoleucine, although less effective than DL-phenylalanine, also counteracted the toxicity of furylalanine, while DL-valine, DL-methionine, and DL-alanine were slightly effective.

For *Escherichia coli*, L-tyrosine and DL-tryptophan were about half as effective as DL-phenylalanine in counteracting the toxicity of furylalanine, while DL-methionine, DL-isoleucine, L-leucine, L-cysteine, L-proline, and L-histidine were slightly active.

The "antiphenylalanine" properties of  $\beta$ -2-furylalanine were found to be quite similar to those of  $\beta$ -2-thienylalanine.

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# THE SYNTHESIS AND MICROBIOLOGICAL PROPERTIES OF TWO ISOMERIC NAPHTHYLALANINES\*

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If, in the indole ring of the tryptophan molecule, the  $\text{—NH—}$  radical is replaced by the vinylene group, the isosteric  $\beta$ -1-naphthylalanine is obtained. As part of a program for the preparation and microbiological investigation of synthetic analogues of natural amino acids, it was decided to study the influence of this compound and of the isomeric  $\beta$ -2-naphthylalanine on the growth of microorganisms.

Both  $\beta$ -1- and  $\beta$ -2-naphthylalanine were first synthesized by Kikkou (1) from the corresponding aldehydes by condensation with hippuric acid, reduction, and subsequent hydrolysis. Sasaki and Kinose (2) obtained  $\beta$ -1-naphthylalanine by the diketopiperazine method (3), but no microbial growth inhibition studies have been reported.

Since 1-chloromethylnaphthalene and 2-bromomethylnaphthalene are easily available, it was thought that application of the recently developed acetamidomalonic ester (4, 5) and acetamidocyanoacetic ester (6) syntheses might result in good yields of the desired naphthylalanines. Accordingly, 1-chloromethylnaphthalene was condensed with ethyl acetamidomalonate in the usual fashion (4, 5). The intermediate was not isolated but was hydrolyzed immediately with 48 per cent hydrobromic acid to give the desired amino acid in 74 per cent yield. In an article appearing after this work was completed, Erlenmeyer and Gfubenmann (7) reported the synthesis of  $\beta$ -1-naphthylalanine through the same intermediate, which was then hydrolyzed with alkali, but these workers claimed that acid hydrolysis was not as satisfactory. Our results would indicate that hydrolysis with 48 per cent hydrobromic acid is as successful as alkaline hydrolysis.

The same amino acid was prepared in equally good yield, by the use of ethyl cyanoacetamidoacetate. The intermediate, isolated in 98 per cent yield, was hydrolyzed by alkali to the amino acid in 78 per cent yield.

Condensation of 2-bromomethylnaphthalene with ethyl acetamidomalonate and subsequent acid hydrolysis of the intermediate gave a 65 per cent yield of  $\beta$ -2-naphthylalanine.

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Neither  $\beta$ -1-naphthylalanine nor  $\beta$ -2-naphthylalanine inhibited the growth of *Escherichia coli*, *Lactobacillus casei*, *Lactobacillus arabinosus* 17-5, two strains of *Neurospora*, or *Saccharomyces cerevisiae*

#### EXPERIMENTAL

*$\beta$ -1-Naphthylalanine Synthesis with Ethyl Acetamidomalonate*—1.15 gm of sodium, purified by being boiled in xylene, were added to 50 ml of absolute ethanol. After the sodium had dissolved completely, 10.9 gm of ethyl acetamidomalonate and 9.2 gm of 1-chloromethylnaphthalene (8) were added. The mixture was refluxed for 2 hours, cooled, and evaporated *in vacuo* to a syrup. The residue was diluted with water and the water decanted after an hour. The material remaining in the flask was boiled with 75 ml of 48 per cent hydrobromic acid for 7 hours. The hydrobromide which separated on cooling the mixture was filtered, dissolved in 100 ml of hot water, treated with charcoal, filtered, neutralized with ammonium hydroxide, and cooled. 8 gm, representing a 66 per cent yield of  $\beta$ -1-naphthylalanine, separated.

*Synthesis with Ethyl Cyanoacetamidoacetate*—To 50 ml of absolute ethanol containing 1.15 gm of sodium and 7.8 gm of ethyl cyanoacetamidoacetate were added 9.2 gm of 1-chloromethylnaphthalene. After being refluxed for 2 hours, the mixture was cooled and evaporated to a small volume *in vacuo*. The residue was stirred with water and the water decanted after an hour, the syrup solidified overnight. On recrystallization from an ethanol-water mixture, 15.8 gm of white crystals, mp 125° (uncorrected), of ethyl  $\alpha$ -acetamido- $\alpha$ -cyano- $\beta$ -(1-naphthyl)-propionate were obtained in 98 per cent yield.

$C_{18}H_{18}N_2O_3$  (310.4) Calculated, N 9.03, found, N 9.14

To hydrolyze the intermediate, 10 gm were refluxed for 17 hours with 80 ml of water containing 10 gm of sodium hydroxide. The solution was cooled, neutralized to pH 5 to 6 with dilute hydrochloric acid, and chilled. The product was purified by treatment with charcoal in acid aqueous solution and recovered after neutralization with ammonium hydroxide, 5.2 gm of  $\beta$ -1-naphthylalanine being obtained. After one more recrystallization from hot water, the substance melted at 240° (capillary), as reported by Kikkou (1), and at 266° within 3 seconds on the Dennis melting point bar.

$C_{17}H_{17}NO$  (215.2) Calculated, N 6.51, found, N 6.44

*Ethyl 2-Naphthylmethylacetamidomalonate*—To 0.89 gm of sodium dissolved in 30 ml of absolute ethanol there were added 8.5 gm of ethyl acetamidomalonate and, in one portion, 8.5 gm of 2-bromomethylnaphthalene (9). Heat was developed immediately. The mixture was refluxed for

2 hours, cooled, evaporated to small volume *in vacuo*, and extracted with water. The yield of ethyl 2-naphthylmethylacetamidomalonate was nearly quantitative, having a melting point of 126° after recrystallization from ethanol, in which it was partially soluble in the cold.

$C_{19}H_{19}NO_5$  (357.4)    Calculated, N 3.96, found, N 4.03

*$\beta$ -2-Naphthylalanine*—12 gm of ethyl 2-naphthylmethylacetamidomalonate were heated under a reflux for 12 hours with 70 ml of 48 per cent hydrobromic acid and filtered. The product was dissolved in 100 ml of hot water, treated with charcoal, filtered, and neutralized with ammonium hydroxide. The amino acid was washed with cold water, ethanol, and ether. In addition to the 4 gm of 2-naphthylalanine obtained in this manner, 0.7 gm was recovered from the hydrobromic acid filtrate, after treatment with charcoal and neutralizing with concentrated ammonium hydroxide, by the addition of alcohol. The pure amino acid had a melting point of 262° when heated rapidly in a capillary tube, and melted at 263–272° within 3 seconds on the melting point bar. This compares with a melting point of 263–264° given by Kikkori (1).

$C_{13}H_{13}NO_2$  (215.2)    Calculated, N 6.51, found, N 6.51

*Microbiological Studies*—The growth of *Escherichia coli* (American Type Culture Collection, strain 9723) and *Saccharomyces cerevisiae* (Fleischmann, strain 139) was not inhibited by either of the two naphthylalanines in concentrations as high as 1 mg per 7.5 ml of culture media. Likewise, these amino acids at concentrations of 1 mg per 10 ml of medium did not inhibit the growth of a wild strain of *Neurospora* (Abbott, strain 4A), and the mutant *Neurospora crassa* (American Type Culture Collection, strain 9278). When *Lactobacillus casei* (American Type Culture Collection, strain 7469) and *Lactobacillus arabinosus* 17-5 were grown in the presence of 5  $\gamma$  of tryptophan, which is enough for about 25 per cent of maximum growth, the addition of these two naphthylalanines did not inhibit the growth.  *$\beta$ -2-Naphthylalanine* did not stimulate the growth of these two organisms, whereas a concentration of 1 mg per 10 ml of medium of the  *$\beta$ -1-naphthylalanine* in the presence of 5  $\gamma$  of tryptophan showed very slight stimulation. 1 mg of  *$\beta$ -1-naphthylalanine* increased the growth equivalent to that obtained with 2 to 5  $\gamma$  of tryptophan.

The authors are indebted to Dr. Herschel K. Mitchell of the California Institute of Technology, who supplied the culture of the wild strain of *Neurospora* (Abbott, strain 4), and to Mrs. Charmion McMillan and Mrs. Hester Purdy McNulty, who carried out the microbiological assays.

## SUMMARY

$\beta$ -1-Naphthylalanine and  $\beta$ -2-naphthylalanine have been synthesized by the acetamidomalonic ester method in 74 and 65 per cent yield, respectively. The two compounds did not inhibit the growth of *Escherichia coli*, *Saccharomyces cerevisiae*, two strains of *Neurospora*, *Lactobacillus casei*, or *Lactobacillus arabinosus*.

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# THE ISOLATION OF HIGHLY POLYMERIZED DESOXY-PENTOSENUCLEIC ACID FROM YEAST CELLS\*

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The nucleic acids occurring in nature are, by convention, divided into two groups, the desoxypentose and the pentose nucleic acids, whose designation emphasizes a difference in their sugar constituents. The extent, however, to which this classification denotes an actual dichotomy rather than a convenient process for the description of a large number of different chemical entities is still subject to conjecture. It appears a matter of real importance to determine whether nucleic acids in general agree with the patterns developed in greater detail for the desoxyribonucleic acid of calf thymus and the ribonucleic acid of yeast. A comparative chemical study of the two nucleic acids isolated from the same cellular material would, in this connection, seem to be one of the most attractive problems.

Among the various microorganisms which can be considered as the starting material for the preparation of desoxypentose nucleic acids, yeast is for several reasons one of the most interesting. As yeast ribonucleic acid has been studied extensively, this would offer an opportunity to compare the two acids from the same source. Moreover, since yeast is one of the most widely explored organisms with respect to its metabolic activities, the general problem of nucleic acid metabolism could be approached, once methods for the isolation from yeast of both types of nucleic acid are made available. Finally, yeast is easily obtained in large quantity.

The presence in yeast cells of definite chromosome-like elements demonstrable by the nucleal stain of Feulgen has been known for some time (1-6). There seems, however, to be only one report in the literature (7) on the preparation from yeast, with the use of strong alkali, of a nucleic acid said to be similar to thymonucleic acid. The evidence is difficult to appraise, since the substance, characterized by a qualitative desoxypentose test and presumably degraded because of the method of isolation,

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† This report is from a dissertation to be submitted by Stephen Zamenhof in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.



was obtained in a yield much higher (about 20 gm per kilo of yeast) than can be reconciled with the figures submitted in the present paper (less than 400 mg per kilo of yeast)

This communication will describe the isolation of the highly polymerized desoxypentose nucleic acid of yeast<sup>1</sup>. A very efficient crushing of the cells proved essential for success. The chemical composition of this substance will be reported later. Since the procedures used avoided diastic operations, material of this kind should lend itself to a number of interesting biological applications.

The methods of isolation employed represent, of course, a combination of many known procedures, but it is the sequence of their application that has proved important in the present study. It must be remembered that yeast cells, containing almost 50 times as much pentose as desoxypentose nucleic acid, probably represent an extreme case as regards the isolation of the latter compound.

The undegraded desoxypentose nucleic acid of yeast is in its physical characteristics reminiscent of the highly polymerized thymonucleic acid isolated by the Hammarsten procedure (8). It is, however, hard to conceive of two macromolecular substances, synthesized in as different cellular systems as calf thymus and yeast, as being identical, although, in the absence of immunological or more refined chemical methods, a strict decision may not yet be possible. (For a recent discussion of some of these problems, compare (9).)

Recent estimates of the desoxypentose nucleic acid content of several microorganisms (staphylococci, typhoid bacilli, *Escherichia coli*) have given values of 3 to 4 per cent of the dry weight, the pentose nucleic acid figures were about twice as high (10). An entirely different distribution is found in yeast, here, desoxypentose nucleic acid amounts to no more than about 0.15 per cent of the dry cells and the ribonucleic acid content is 30 to 50 times as high. This wide variation within unicellular organisms suggests that the desoxypentose nucleic acid molecules of the cell may not all have the same biological function.

## EXPERIMENTAL

### *Isolation of Desoxypentose nucleic Acid*

*Extraction*<sup>2</sup>—1 kilo of fresh bakers' yeast was washed with 1 liter of 0.1 M sodium citrate (pH 7.3). The yeast cells, recovered by centrifugation,

<sup>1</sup> In the course of the present studies the observation was made that commercial yeast nucleic acid contained about 2 per cent of depolymerized desoxypentose nucleic acid. A supplementary description of the procedure for the preparation of such material which, although degraded, may be of interest for chemical studies on yeast desoxypentose nucleotides also has been included here.

<sup>2</sup> The experiments were carried out in the cold and at an about neutral pH.

were suspended in 180 cc of the sodium citrate solution<sup>3</sup> and the thick suspension was passed through an ice-cooled wet crushing mill for bacteria (13), constructed by Unicam Instruments, Ltd, Cambridge, England. Each 50 cc portion was ground for 30 minutes. Following dilution with 590 cc of sodium citrate, the crushed suspension was centrifuged for 2 hours at 4000 R P M. Two solid layers sedimented underneath a very opalescent supernatant (915 cc),<sup>4</sup> a bottom layer of intact cells and an upper layer of cellular fragments. The microscopic examination of the stained (5) debris revealed the presence of chromosome-like bodies closely resembling the elements demonstrable by the Feulgen technique in intact yeast cells. A separate determination showed that about 55 per cent of the cells had actually been crushed.

The upper solid layer consisting of cellular fragments (485 cc) was suspended in 850 cc of ice-cold M sodium chloride solution of pH 6.3 (14, 15). The slimy mixture was kept in the refrigerator for 72 hours and then centrifuged at 4000 R P M for 2 hours.<sup>5</sup> The rapid addition of 2 volumes of chilled absolute ethanol to the very viscous supernatant<sup>6</sup> resulted in the precipitation of white threads that could easily be wound on a glass rod and thereby separated from a granular precipitate suspended in the mother liquor. The threads were washed thoroughly by successive immersion in three portions of 73 per cent ethanol, drained, and redissolved in 300 cc of M sodium chloride with the use of a high speed mixer.

The turbid solution was freed of protein by being stirred in a high speed mixer with one-third of its volume of a 9:1 mixture of chloroform-octyl alcohol (16) for 5 minutes, followed by centrifugation at 4000 R P M for 1 hour. After eight treatments the solution was free of protein and gave no biuret test (17).<sup>7</sup> At this stage, it was found to contain 0.6 mg of desoxypentose nucleic acid per cc, corresponding to a total of 180 mg in the original 300 cc of solution.

The addition of 2 volumes of ethanol to the clear protein-free solution

<sup>3</sup> The use of sodium citrate is based on the observation that desoxyribonuclease requires magnesium ion (11, 12) and can be inhibited by sodium citrate (12).

<sup>4</sup> A study of some of the components of the supernatant, carried out in collaboration with Dr. M. A. Nyman, will be presented later.

<sup>5</sup> It hardly pays to reextract the sediment with M sodium chloride, the recovery of an additional 8 per cent of the total desoxypentose nucleic acid content is set off by the introduction of more contaminating material.

<sup>6</sup> The viscous sodium chloride solution appears sensitive to temperature. From a solution stored at room temperature for several hours, long fibers can no more be obtained by precipitation with alcohol.

<sup>7</sup> The reextraction with M NaCl of the combined chloroform gels resulting from the deproteinization, followed by the renewed treatment with chloroform of the extract, yielded some additional desoxypentose nucleic acid, this, however, amounted only to 6 per cent of the recoverable total.

again produced white threads that were spooled on a rod, as described before. An additional amount of fibrous nucleic acid could be recovered by reworking the granular precipitate remaining in the mother liquor.

*Purification*—When the threads obtained by the above procedure were analyzed, they were found to contain only 19 per cent of desoxy-pentose-nucleic acid, the remainder consisted of ribonucleic acid (64 per cent) and of a polysaccharide whose principal constituent seemed to be mannose, as shown by a color reaction recently described (18). The best procedure for the purification was found to be one that made use of the difference in solubility of the calcium salts of the two nucleic acids. It was in part patterned after the method developed for the separation of the pneumococcus-transforming agent from pneumococcal polysaccharide (19). An electrophoretic separation method also was found possible, but it was accompanied by much greater losses.<sup>8</sup>

The well drained threads were taken up in 20 cc of neutral 10 per cent aqueous calcium chloride and the viscous milky solution was clarified by centrifugation at 20,000 R.P.M. for 2 hours. The sediment was twice washed, under the same centrifugal conditions, with 6 cc portions of 10 per cent  $\text{CaCl}_2$ . The slow addition of 0.2 to 0.3 volume of cold absolute ethanol to the combined clear supernatants (32 cc) brought about the separation of white fibers that were lifted in the usual manner and washed twice with 10 per cent calcium chloride solution containing 0.3 volume of ethanol.

This fraction was contaminated with about 20 per cent of ribonucleic acid which could be removed by enzymatic digestion. To a solution of the precipitate in 45 cc of 0.2 M sodium borate buffer of pH 7.8, 1.5 mg of crystalline ribonuclease<sup>9</sup> were added. The solution was subjected to dialysis at room temperature against two changes of 2 liter portions of the borate buffer for 14 hours, against running tap water for 17 hours, and, finally, against several changes of ice-cold distilled water for 26 hours. Then it was again deproteinized, as described before, and evaporated in

<sup>8</sup> These experiments are perhaps of interest by providing an example of the difference in electrophoretic mobilities (compare (20)) of the two nucleic acids which in this case were derived from the same source. When the mixture was subjected to electrophoresis in 0.02 M phosphate buffer of pH 7.4 (containing 0.15 M NaCl), three components could be discerned with the following descending mobilities: I,  $-16.0 \times 10^{-5}$ , II,  $-12.1 \times 10^{-5}$ , III, almost stationary. The electrophoretic separation yielded two fractions: the faster component consisted of desoxy-pentose-nucleic acid with an admixture of only 13 per cent of ribonucleic acid, and the slower component (II) was mainly ribonucleic acid with about 18 per cent of the desoxy-pentose compound. We are grateful to Dr. D. H. Moore for these and other electrophoresis experiments.

<sup>9</sup> We are greatly indebted to Dr. M. Kunitz of the Rockefeller Institute, Princeton, New Jersey, for this enzyme preparation.

the frozen state in a vacuum (If the preparation is to be used for biological experiments, it may be preferable to precipitate it with alcohol from its solution in the presence of an electrolyte, such as sodium acetate, instead of recovering it by the evaporation of the frozen solution)

The sodium salt of desoxypentosenucleic acid thus obtained weighed 105.5 mg. It formed a white fluff which was readily soluble in water, giving a clear viscous solution. The biuret reaction was negative.

*Analytical Composition*—The desoxypentosenucleic acid content was determined colorimetrically by means of the reaction with diphenylamine (21) or with cysteine (22). A particularly well purified sample of potassium desoxyribonucleate of calf thymus, prepared by a procedure to be described later, served as the standard. The ribonucleic acid content was estimated by means of the orcinol reaction (23), correction being made for the color produced with orcinol by the desoxy sugar. A purified preparation of yeast ribonucleic acid was used as the standard.

Colorimetric methods served for the estimation of the hexose content (24) of the small amount of polysaccharide impurity, probably mainly mannan which is known to occur in the yeast cell wall, and of the phosphorus content (25). Nitrogen was determined by the micro-Kjeldahl method.

The particular preparation of the sodium salt of desoxypentosenucleic acid whose isolation from yeast has been described in detail in this paper had the following composition: N 14.0, P 8.3, desoxypentosenucleic acid 91, ribonucleic acid 3.4 per cent. The N/P percentage ratio, therefore, was 1.69, the atomic ratio 3.73. As was already reported in part in a preliminary note (26), different preparations varied between the following percentage figures: N 13.3 to 14.0, P 8.3, desoxypentosenucleic acid 86 to 91, ribonucleic acid 3.1 to 5.6, polysaccharide (mannan) 5 to 10.

*Yield*—If account is taken of the facts reported before, namely that about 55 per cent of the 1000 gm. of yeast processed actually had been crushed and that the original extract obtained comprised 180 mg. of desoxypentosenucleic acid, the yield in pure sodium desoxypentosenucleate amounted to 53 per cent of the total. An additional 20 per cent of nucleic acid may be recovered by reworking the granular precipitates, as was mentioned before. The total amount of desoxypentosenucleic acid extracted under the preparatory conditions described corresponded to 0.033 per cent of the wet weight of the crushed cell portion.

#### *Physical Properties*

*Ultraviolet Absorption*—The absorption spectrum, determined with a 0.006 per cent solution of the sodium desoxypentosenucleate in distilled water by means of a Beckman photoelectric quartz spectrophotometer, is

reproduced in Fig 1 The manner in which the extinction values are plotted as the ordinate requires explanation When more data on nucleic acids of different origin become available, a comparison of their extinction characteristics will be of importance Since the particle weights of these macro molecules are known only vaguely and preparations of different degrees of purity may have to be compared, the best expression would appear to be one that is based on the P content of the particular substance For this reason, the values were plotted as  $\epsilon(P)$ , which may be defined as

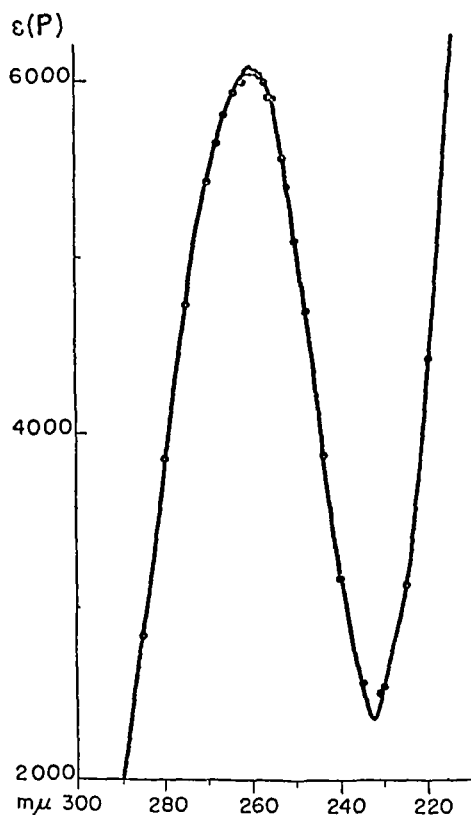


FIG 1 Ultraviolet absorption of yeast sodium desoxypentosenucleate in distilled water

the atomic extinction coefficient with respect to phosphorus, viz  $\epsilon(P) = 30.98E/cl$ , where  $E$  is the extinction,  $c$  the phosphorus concentration of the solution in gm per liter, and  $l$  the thickness of the absorbing layer in cm

The sodium salt of desoxypentosenucleic acid exhibited an absorption maximum at 260  $m\mu$ , a minimum at 232  $m\mu$ , with  $\epsilon(P)$  values of 6056 and 2485 respectively Another preparation had a maximum  $\epsilon(P)$  value of 6210 at 260  $m\mu$

*Electrophoretic Mobility*—When examined in the Tiselius cell at 15°, the solution of the substance in 0.02 M phosphate buffer (containing 0.15 M NaCl) of pH 7.4 exhibited one sharp peak with an ascending mobility of  $-15.7 \times 10^{-5}$  sq cm per volt per second.

*Viscosity*—The determinations were carried out in Ostwald-Fenske viscosity pipettes (water value 10.8 seconds) at 30.3°. The specific viscosities,  $\eta_{sp}$ , for different preparations (with the percentage concentration in distilled water given in parentheses) were 3.7 (0.09), 5.6 (0.12), and 5.9 (0.11).

Of one preparation, the specific viscosities (2.7 to 0.2) of a series of very dilute solutions were determined. When the value of 0.55 cc per gm for the apparent specific volume was used, which has been reported for thymonucleic acid (27), the viscosity increments could be calculated and plotted against the volume fractions of the solute. In this manner a viscosity increment of 7500 for zero concentration was obtained by extrapolation which, if the equation of Kuhn (28) is employed, would correspond to an axial ratio of about 350. This value, certainly open to more than the usual objections, will at least indicate the order of magnitude.

*Diffusion*—The diffusion constant was measured in diffusion cells that differed mainly by their smaller size from those described by Northrop and Anson (29). Three cells, previously calibrated by means of a 0.1 M potassium chloride solution (30, 31), were filled with a 0.19 per cent solution of the sodium salt of the yeast desoxypentosenucleic acid in distilled water. A fourth cell, similarly calibrated, contained a solution of sodium thymonucleate (8) of the same concentration.

The diffusion was permitted to proceed against distilled water at 20° for 7 days, when the outside fluids were concentrated *in vacuo* to a small volume and analyzed (21) for their desoxypentosenucleic acid contents. The diffusion constants found were 1.11, 1.09, and 1.07, giving an average value of  $D_{20} = 1.1 \times 10^{-7}$  sq cm per second for the nucleic acid from yeast. The same value,  $D_{20} = 1.1 \times 10^{-7}$ , was found for thymonucleic acid.<sup>10</sup>

#### *Estimation of Total Extractable Desoxypentosenucleic Acid*

When a suspension of 30 gm of bakers' yeast in citrate solution was thoroughly crushed by being passed through the bacterial mill for 70 minutes, dry weight determinations of the several fractions showed that 25.5 gm (85 per cent of the cells), corresponding to a dry weight of 7.61 gm, had been disintegrated. The extraction of the cell debris with two portions of M sodium chloride, the precipitation with ethanol, and the de-

<sup>10</sup> The diffusion behavior of thymonucleic acid has been studied by Tennent and Vilbrandt (27).

proteinization, as described above, yielded a solution containing (22) a total of 11.3 mg of desoxypentose nucleic acid. This corresponds to a minimum content of 0.044 per cent of the wet cells or of 0.15 per cent of the dry weight. Jeener and Biachet (32) recently estimated the content as 0.03 per cent of the wet weight.

The ribonucleic acid content of dry bakers' yeast has been reported as around 6.9 per cent (33), *i.e.*, about 46 times as high as the desoxypentose nucleic acid content submitted here. Whether the amount of desoxypentose nucleic acid is changed for yeast cells in the budding stage is not yet known.

The average wet weight of a yeast cell, determined by microscopical measurements on living cells and by the estimation of the number of cells per gm of yeast cake, was found as  $7.5 \times 10^{-8}$  mg. Each cell contained, therefore, an average of  $3.3 \times 10^{-11}$  mg of desoxypentose nucleic acid, *i.e.*, about one-thirtieth of the amount reported for thymus cells (34).

#### *Preparation of Depolymerized Desoxypentose nucleic Acid from Commercial Yeast Nucleic Acid*

A suspension of 40 gm of yeast nucleic acid (Schwarz Laboratories, Inc., New York) in 300 cc of distilled water was agitated in a high speed mixer for 5 minutes and the insoluble residue reextracted in the same manner. The combined supernatants (90 minutes at 4000 R.P.M.), made strongly alkaline (pH 13.8) with 30 per cent sodium hydroxide, were kept overnight at 30°. The addition of 2 volumes of ethanol to the neutralized solution produced a precipitate which was dissolved in 40 cc of alkali and again kept overnight at pH 13.8. The solution, adjusted to pH 7.5 with acetic acid, deposited crude sodium guanylate which was centrifuged off. The supernatant was subjected to dialysis against running water for 72 hours which removed most of the ribonucleotides. The solution was made 1 M with respect to sodium acetate and the crude sodium desoxypentose nucleate precipitated by the addition of 2 volumes of ethanol. The precipitate was washed with 80, 95 per cent, and absolute alcohol and with ether and dried *in vacuo*. It weighed 165 mg (0.4 per cent of the yeast nucleic acid used) and formed a light brown, hygroscopic powder containing N 12.1, P 8.5, desoxypentose nucleic acid 79, and ribonucleic acid 6 per cent.

#### SUMMARY

The isolation from yeast cells of highly polymerized desoxypentose nucleic acid by a procedure avoiding drastic operations and enzymatic degradation is described. Some of the physical characteristics of this substance, demonstrating its high degree of asymmetry, are discussed. The total

amount of desoxyribonucleic acid extractable from bakers' yeast is estimated as 0.044 per cent of the wet cells. A brief description of the preparation from commercial yeast nucleic acid of a specimen of depolymerized desoxyribonucleic acid is included.

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# N-ACYLATED AND N-METHYLATED GLYCYLDEHYDROALANINE

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Earlier studies have shown that a variety of synthetic dehydropeptides are enzymatically hydrolyzed in aqueous extracts of several animal and plant tissues (1-4). The substrates used were dipeptides, in which dehydroalanyl or dehydrophenylalanyl residues were N-acylated with glycyl, chloroacetyl, or acetyl radicals.

Glycyldehydroalanine is rapidly hydrolyzed in digests of all tissue preparations studied, yielding equivalent amounts of ammonia and pyruvic acid (2). This substrate possesses a free  $\alpha$ -amino group on the glycyl radical. The purpose of the present study is to observe any relation between the integrity of this group and the susceptibility of the substrate to enzymatic attack. Chloroacetylglycyldehydroalanine, glycylglycyldehydroalanine, N-methylglycyldehydroalanine, and chloroacetyl-N-methylglycyldehydroalanine were synthesized and studied in certain rat tissue digests.

In the course of the synthetic studies, the unexpected observation was made that methylamine or dimethylamine reacting in excess with chloroacetyldehydroalanine would, under the circumstances, not only replace the halogen but also substitute at the double bond.

## EXPERIMENTAL

*Chloroacetylglycyldehydroalanine*—14.4 gm of glycyldehydroalanine (4) were dissolved in 50 cc of chilled 2 N NaOH, and with further chilling treated alternately in four portions with 13.5 gm of freshly distilled chloroacetyl chloride and 60 cc of 2 N NaOH. The mixture was brought to pH 2 by addition of 5 N HCl and evaporated *in vacuo* to half of its volume. The product crystallized from the concentrate on chilling and scratching. Yield, 12.5 gm. It was twice crystallized from the minimum amount of hot water, from which it separates on cooling in the form of long needles, m p 177° with decomposition.

$C_7H_9O_4N_2Cl$  Calculated, N 12.7, Cl 16.1, found, N 12.4, Cl 15.8

*Glycylglycyldehydroalanine*—3 gm of chloroacetylglycyldehydroalanine were dissolved in 30 cc of ammonia water saturated at 5°, and the solution allowed to stand at 25° for 3 days. The solution was then evaporated to dryness *in vacuo* and the residue triturated with absolute alcohol to remove ammonium chloride. The residue was taken up in the minimum amount

of hot water, and the product precipitated by addition of a moderate excess of absolute alcohol. Recrystallization of the compound by dissolving in hot water followed by addition of hot alcohol resulted in the separation of long glistening prisms, m p  $184^{\circ}$  with decomposition.

$C_7H_{11}O_4N_3$  Calculated, C 41.8, H 5.5, N 20.9, found, C 41.5, H 5.5, N 20.4

*N*-Methylglycyldehydroalanine—50 gm of chloroacetyldehydroalanine (4) were dissolved in 500 cc of chilled 25 per cent aqueous methylamine, and the resulting solution kept at  $0-5^{\circ}$  for 48 hours. At the end of this period the solution was evaporated *in vacuo*, at a temperature never higher than  $20^{\circ}$ , to a thick syrup. The syrup was taken up in 100 cc of cold distilled water, treated with 10 cc of glacial acetic acid, and 1 liter of absolute alcohol was added. *N*-Methylglycyldehydroalanine crystallized from the mixture as flat prisms. After filtering, washing with alcohol and ether, and finally drying, the product weighed 14 gm. It was recrystallized from a mixture of hot water and alcohol from which, on cooling, it separated in glistening, large prisms, m p  $177^{\circ}$  with decomposition.

$C_6H_{10}O_3N_2$  Calculated, C 45.6, H 6.3, N 17.7, found, C 45.5, H 6.3, N 17.6

At higher temperatures, some substitution of methylamine at the double bond of the dehydropeptide apparently occurs. When 50 gm of chloroacetyldehydroalanine were dissolved in excess aqueous methylamine as above, with no attempt at external cooling, the solution became quite warm. The solution was allowed to stand at  $25^{\circ}$  for 4 days, and was then evaporated *in vacuo* at  $40^{\circ}$  to a thick, yellowish oil. The oil was taken up with absolute alcohol once or twice and evaporated again in a boiling water bath. On shaking with an excess of absolute alcohol in the cold for several hours, the oil became granular and could be readily filtered. After washing with alcohol and ether, the dried product weighed 35 gm. It was purified by dissolving in the minimum amount of cold water, filtering, and adding absolute alcohol in moderate excess to the filtrate. A white oil separated which rapidly crystallized in sheaves of long, narrow prisms. Yield, 28 gm, m p  $183^{\circ}$  with decomposition.

Aqueous solutions of the compound are slightly acid to litmus and alkaline to Congo red. Such solutions do not decolorize added bromine, and show only a slight, general, and non-specific absorption in the ultraviolet. A heavy precipitate of silver chloride occurs when the solutions are treated with nitric acid and silver nitrate. Elemental analysis indicates that not only was the chlorine atom in chloroacetyldehydroalanine substituted by a methylamino residue but that another methylamino residue was added to the compound as well. A monohydrochloric acid salt would consequently

be formed. The close agreement between the observed analytical data and those calculated for such a compound is revealed as follows

$C_8H_{15}O_2N_3 + HCl$	Calculated	C 37.2, H 7.1, N 18.7, Cl (ionic) 15.7
	Found	" 37.1, " 6.6, " 18.4, " " 15.5

The methylamino residue might saturate the double bond of chloroacetyldehydroalanine in two ways, (1) by forming an  $\alpha$ -N-methylglycylamino- $\alpha$ -N-methylaminopropionic acid hydrochloride or (2) by forming an N-methylglycyl- $\alpha$ -amino- $\beta$ -methylaminopropionic acid hydrochloride. Both compounds would yield the same analytical results. It is improbable that the preparation could be related to the former compound because of the instability of such types of compounds in water, whereby pyruvic acid and ammonia are rapidly formed (5). The probability that the preparation would be described by the latter alternative arises from the fact that whereas the intact preparation yields no nitrogen after treatment with nitrous acid for 5 minutes the hydrolysate obtained after boiling 75 micromoles of the preparation in 2 N HCl for 2 hours yields 95 per cent of the equivalent amount of  $\alpha$ -amino nitrogen (Van Slyke). No ammonia, methylamine, or pyruvic acid was present in the hydrolysate, and the mole of  $\alpha$ -amino nitrogen released per mole of compound must have been derived from the hydrolysis of a single, saturated peptide bond.

In our experience, when chloroacetyldehydroalanine is treated with an excess of aqueous ammonia, whether at 5° or at 25°, glycyldehydroalanine in a yield of close to 80 per cent is invariably formed. Treatment of chloroacetyldehydroalanine at low temperatures with an excess of methylamine leads to the formation of N-methylglycyldehydroalanine in yields of about 20 to 30 per cent, and at higher temperatures to yields of 50 to 70 per cent of a saturated peptide whose composition and properties appear to relate it to N-methylglycyl- $\alpha$ -amino- $\beta$ -methylaminopropionic acid hydrochloride. When chloroacetyldehydroalanine is treated with an excess of an aqueous solution of dimethylamine, whether at 5° or at 25–40°, there is formed a saturated compound in about a 40 to 60 per cent yield (depending upon the temperature), whose composition and properties appear to relate it to an N,N-dimethylglycyl- $\alpha$ -amino- $\beta$ -dimethylaminopropionic acid hydrochloride. A typical preparation is as follows.

11 gm of chloroacetyldehydroalanine were dissolved in 120 cc of chilled 30 per cent aqueous dimethylamine, and the solution kept for 48 hours in the ice chest at 5°. It was then evaporated to a low bulk *in vacuo* at 20°, and the residue was treated with an excess of absolute alcohol. The precipitate was filtered, dissolved in water, and crystallized by the careful addition of alcohol. Repetition of the procedure yielded 5.5 gm of a prepa-

ration which appeared in the form of long prisms, m p 155° with decomposition

$C_9H_{19}O_3N_2 + HCl$	Calculated	C 42.6, H 7.8, N 16.6, Cl (ionic) 14.0
	Found	" 42.7, " 7.8, " 16.3, " " 13.8

Aqueous solutions of the compound are slightly acid to litmus and alkaline to Congo red. Such solutions do not decolorize added bromine, and show only a slight, general, and non-specific absorption in the ultraviolet. The intact preparation yields no nitrogen after treatment with nitrous acid for 5 minutes, but, as in the case of the methylamine substitution product, the hydrolysate obtained after boiling 75 micromoles of the preparation in 2 N HCl for 2 hours yields 95 per cent of the equivalent amount of  $\alpha$ -amino nitrogen (Van Slyke).

The ease of addition or substitution of such simple amines at the double bond of the dehydropeptide was quite unexpected. More exact studies of the conditions under which this phenomenon occurs, and the rates at which the reaction proceeds, will be the subject of a future communication.

*Chloroacetyl-N-methylglycyldehydroalanine*—15.8 gm of N-methylglycyldehydroalanine were dissolved in 50 cc of 2 N NaOH, chilled, and treated alternately with 13 gm of freshly distilled chloroacetyl chloride and 60 cc of 2 N NaOH. The mixture was acidified to a Congo blue reaction with 5 N HCl and evaporated to one-half its volume *in vacuo*. On chilling and scratching, a voluminous crystallization of the product occurred. The compound was crystallized twice from hot water, from which it separates in the form of long needles. Yield, 8.2 gm, m p 152° with decomposition.

$C_8H_{11}O_4N_2Cl$	Calculated	C 40.9, H 4.7, N 11.9, Cl 15.2
	Found	" 40.9, " 4.6, " 12.0, " 15.1

Attempts to prepare the corresponding glycyl-N-methylglycyldehydroalanine by treatment of the chloroacetyl derivative with excess ammonia were unsuccessful. A yellowish red gum was obtained from which no crystalline material could be derived.<sup>1</sup>

*Ultraviolet Absorption Spectra*—All of the acylated and methylated dehydropeptides prepared possess the characteristic absorption in the ultraviolet of this class of compounds (3, 4) (Fig. 1). The band at 2400 Å for the substituted dehydropeptides is not so pronounced as for glycyldehydroalanine since, with increasing length of the peptide chain, there is more absorption at the shorter wave-lengths.

*Enzymatic Studies*—The peptides were incubated with buffered, fresh aqueous rat tissue extracts as described earlier (4). The digests consisted

<sup>1</sup> Chloroacetyl-glycine and chloroacetyl-glycylglycine were prepared and purified as described (6).

of 1 cc of aqueous tissue extract, plus 2 cc of 0.15 M borate buffer at pH 8.1, plus 1 cc of either water or 0.025 M substrate solution. Stock solutions of the substrates were brought to pH 7.0 by addition of dilute NaOH. The substrates were completely stable in the presence of boiled extracts. Dehydropeptidase activity was measured as before in terms of the micromoles  $\times 10$  of ammonia-N evolved over the extract blanks per hour per mg of total N in the extracts (4). No ammonia was evolved from the glycyl resi-

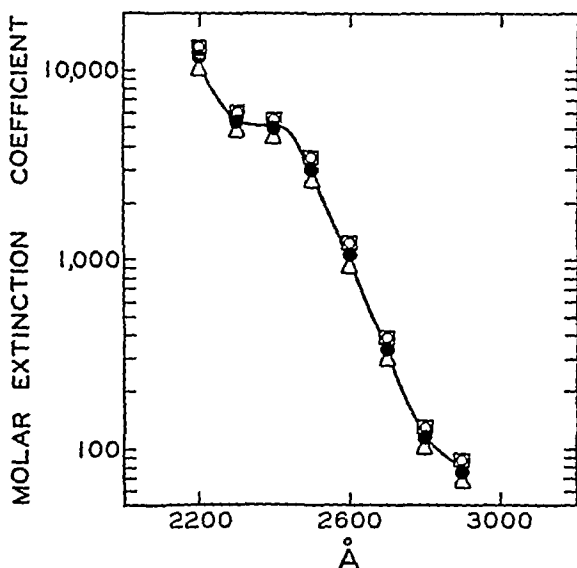
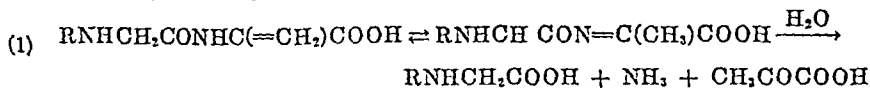


Fig. 1. Ultraviolet absorption spectra of compounds at  $1.7 \times 10^{-4}$  M concentration in water: □, chloroacetyl-glycyldehydroalanine; Δ, glycylglycyldehydroalanine; ○, chloroacetyl-N-methylglycyldehydroalanine; ●, N-methylglycyldehydroalanine.

dues. The ammonia measured was derived from the hydrolysis of the terminal dehydroalanyl residue as follows:



where R may be acyl, methyl, or hydrogen. The data are given in Table I.

The hydrolysis of chloroacetyl-glycyldehydroalanine and of glycylglycyldehydroalanine in the various tissue preparations studied occurs at very nearly the same rate as that of glycyldehydroalanine but not that of chloroacetyldehydroalanine. N-Methylglycyldehydroalanine is hydrolyzed at a rate markedly less than that of glycyldehydroalanine, while chloroacetyl-N-methylglycyldehydroalanine is apparently not affected in extracts of any of the tissues studied.

A critical compound in the interpretation of the data is chloroacetyl-glycyldehydroalanine. Whereas it is conceivable that glycylglycyldehydroalanine may be rapidly attacked by an aminopeptidase in tissue extract preparations, which leads to the primary hydrolysis of a terminal glycyl residue with the production of glycyldehydroalanine (*cf* (1)), no such enzymatic hydrolysis of a chloroacetyl residue appears probable. Attention was therefore largely devoted to the chloroacetyl derivatives. When chloroacetyl-glycine or chloroacetyl-glycylglycine was incubated with rat kidney extracts under conditions employed with chloroacetyl-glycyldehydroalanine (extract containing 60  $\gamma$  of total N per cc, incubation period of 15 minutes at 37°) (Table I), no increment in  $\alpha$ -amino nitrogen could be observed. Furthermore, no increment in  $\alpha$ -amino nitrogen could be observed in digests

TABLE I  
*Action of Dehydropeptidases on N-Acylated Dehydropeptides\**

Substrate†	Micromoles $\times$ 10 ammonia N evolved per hr per mg total N in digests with					
	Kidney	Liver	Pan- creas	Brain	Spleen	Muscle
N-Methylglycyldehydroalanine	800	25	280	30	166	16
Chloroacetyl-glycyldehydroalanine	1580	50	450	40	240	28
Glycylglycyldehydroalanine	1660	60	500	64	320	35
Chloroacetyl - N - methylglycyldehydro- alanine	0	0	0	0	0	0

\* Digests consisted of 1 cc of fresh aqueous rat tissue extract, plus 2 cc of 0.15 M borate buffer at pH 8.1, plus 1 cc of either water or 0.025 M substrate solution. Chloroacetyl peptide solutions were carefully neutralized to pH 7.0 with dilute NaOH before use. Incubation temperature, 37°.

† The corresponding values for glycyldehydroalanine and for chloroacetyldehydroalanine are, respectively, with kidney 1620 and 100, liver 60 and 28, pancreas 530 and 10, brain 72 and 0, spleen 331 and 0, muscle 38 and 0 (4).

of chloroacetyl-glycyldehydroalanine with rat kidney which had been allowed to go to complete splitting of the dehydropeptide bond as measured by the loss in absorption at 2500 Å (4) and from which the ammonia had been completely removed by an alkaline aeration procedure. If the chloroacetyl residue had been hydrolyzed from the glycyl residue, the latter, after the hydrolysis of the dehydropeptide bond, would have yielded an equivalent amount of amino nitrogen. Kidney was selected as the tissue for these experiments because of its high activity of various peptidases.

#### DISCUSSION

The fact that no increase in  $\alpha$ -amino nitrogen appears in ammonia-free kidney digests of chloroacetyl-glycyldehydroalanine suggests that the mole-

cule is hydrolyzed at the dehydropeptide bond exclusively under the conditions studied. Furthermore, the equally important fact that the rate of hydrolysis of this substrate, not only in kidney but in extracts of other tissues as well, approximates that of glycyldehydroalanine suggests that this compound is hydrolyzed by dehydropeptidase I<sup>2</sup>. It was believed earlier (2) that the proper substrate for this enzyme would be glycyldehydroalanine or some analogous compound with a free  $\alpha$ -amino group adjacent to the susceptible dehydropeptide bond. It appears from the present results that the presence of a free, basic  $\alpha$ -amino group in the substrate is not necessarily a *sine qua non* for dehydropeptidase I activity. What does, however, appear to be a necessary condition for the activity of this enzyme is the presence of an  $\alpha$ -nitrogen atom to which at least 1 hydrogen is attached. The substrate conditions for dehydropeptidase I activity are thus described by equation (1) above. Chloroacetyl-N-methylglycyldehydroalanine, with no hydrogen in the  $\alpha$ -nitrogen atom, is not susceptible to enzymatic hydrolysis (Table I). The stability of this compound in rat kidney extracts means that it is resistant not only to the action of dehydropeptidase I, but also to that of dehydropeptidase II. Dehydropeptidase II acts upon such substrates as chloroacetyldehydroalanine and acetyldehydroalanine, not upon glycyldehydroalanine, and it would appear from the available data that the action of this enzyme is blocked by the presence of an  $\alpha$ -nitrogen atom adjacent to the dehydropeptide bond, whether that nitrogen is basic or not.

The reduced effectiveness of N-methylglycyldehydroalanine as a substrate, compared with that of glycyldehydroalanine, may be explicable on the basis of a steric effect in the former compound. On such a basis, the N-ethyl, N-propyl, etc., derivatives of glycyldehydroalanine should be increasingly ineffective substrates, and work on this topic is now in progress. As noted above, the synthesis of N,N-dimethyl and, presumably, the corresponding N,N-dialkyl derivatives of glycyldehydroalanine is rendered difficult because of the possibility of substitution of the amines at the double bond of the dehydropeptide.

#### SUMMARY

Chloroacetylglycyldehydroalanine, glycylglycyldehydroalanine, chloroacetyl-N-methylglycyldehydroalanine, and N-methylglycyldehydroalanine were prepared, and their susceptibility to the action of extract preparations of various rat tissues was studied. Chloroacetylglycyldehydroalanine and glycylglycyldehydroalanine were hydrolyzed in digests of all tissues studied

<sup>2</sup> This is more clearly shown by the action of the purified dehydropeptidase I of Shack (personal communication) which attacks chloroacetylglycyldehydroalanine at close to the same rate as glycyldehydroalanine. Neither substrate is appreciably hydrolyzed by purified dehydropeptidase II.



at close to the same rate as glycyldehydroalanine. In the case of chloroacetylglycyldehydroalanine with kidney digests, no increase in  $\alpha$ -amino nitrogen was noted in the ammonia-free digests after the dehydropeptide bond was completely hydrolyzed. N-Methylglycyldehydroalanine was hydrolyzed at a lower rate than glycyldehydroalanine, while chloroacetyl-N-methylglycyldehydroalanine was not hydrolyzed in any of the tissues studied. From the data obtained, certain substrate specificities for the action of dehydropeptidases I and II have been considered.

A brief description is given of the action of aqueous solutions of methylamine and of dimethylamine on chloroacetyldehydroalanine, leading under certain experimental circumstances to apparent substitution of the amines at the double bond of the dehydropeptide.

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# THE INTRACELLULAR DISTRIBUTION OF PROTEIN, NUCLEIC ACIDS, RIBOFLAVIN, AND PROTEIN-BOUND AMINOAZO DYE IN THE LIVERS OF RATS FED *p*-DIMETHYLAMINOAZOBENZENE\*

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Recent studies have shown that the formation of liver tumors in rats fed *p*-dimethylaminoazobenzene is preceded by the accumulation of aminoazo dyes firmly bound to the liver protein (1). The bound dyes have been freed from the liver protein only by enzymatic or alkaline hydrolysis, the major fraction of the released dye possesses highly polar properties and is therefore not the dye fed. The protein-bound dyes are found only in the site of tumor formation, the liver, and only in the livers of the two known susceptible species, *e.g.*, rats and mice. The latter species is less susceptible to the dye than rats and less bound dye is found in their livers. *p*-Monomethylaminoazobenzene, a carcinogenic metabolite of the parent dye, gives rise to the same levels of bound dye, while the non-carcinogenic metabolite, *p*-aminoazobenzene, yields only low levels of a different bound dye. A high level of dietary riboflavin, which reduces the carcinogenicity of the parent dye, also decreases the level of protein-bound dye in the liver. The bound dyes are not found in the liver tumors. These observations indicate that the protein-bound dyes play an important but as yet undefined rôle in the carcinogenic process induced by *p*-dimethylaminoazobenzene.

We have now applied the technique of differential centrifugation to determine the intracellular distribution of protein-bound dye and other constituents in the livers of rats fed diets high and low in riboflavin and with and without *p*-dimethylaminoazobenzene. The general procedure described by Claude (2) was adapted<sup>1</sup> to resolve homogenates of the livers into four fractions: nuclei, large granules (mitochondria), small granules (microsomes), and the supernatant fluid from which these particles have been sedimented. The homogenates were made in hypertonic sucrose solution, since the recent work by Hogeboom, Schneider, and Pallade

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<sup>1</sup> We are grateful to Dr. W. C. Schneider for advice on the fractionation procedures used in this paper.

(3, 4) showed that this medium prevents the agglutination of the large granules and preserves their morphological characteristics. Each fraction and the original homogenate were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye.

### Methods

Four groups of female Sprague-Dawley rats weighing 160 to 200 gm were fed the diets *ad libitum* for 4 weeks. The basal diet was the semi-synthetic one previously described (5) and was modified to contain 0.5 or 20.0 mg of riboflavin per kilo with either no azo dye or 0.06 per cent *p*-dimethylaminoazobenzene. 90 to 100 per cent of the rats fed this level of dye in the low riboflavin diet for 4 months develops liver tumors (5, 6). Two livers were used for each fractionation. The rats were killed with ether, the inferior vena cava exposed, and an L-shaped hypodermic needle inserted into the vein cephalic to the liver and clamped in place by a rubber-tipped hemostat. A second hemostat was placed on the inferior vena cava caudal to the liver. After severing the portal vein the liver was perfused with 30 ml of 0.14 M NaCl under a hydrostatic pressure of 13 cm. The excised liver was then forced through an all-plastic (Plexiglas) tissue mincer (a thick walled tube with a tightly fitting screw-driven piston which forced the parenchyma through a disk containing numerous 0.8 mm holes). This operation facilitated homogenization by removing much of the connective tissue, blood vessels, and bile ducts. The minced tissue was collected in 0.88 M sucrose solution buffered to pH 7.6 with 0.01 M phosphate and further diluted to make a 12 per cent suspension (final pH 7.4) by weight. The mixture was then homogenized (7) for 15 minutes in a 1 × 8 inch test-tube with a tightly fitting Plexiglas pestle driven by a stainless steel shaft. The entire procedure was carried out at 0–3°.

The differential centrifugations were carried out in a refrigerated International centrifuge, model PR, size 1, with the multispeed attachment and No. 295 head carrying six cellulose acetate tubes with a total capacity of 72 ml. The temperature of the tube contents did not exceed 5° on short runs or 10° on long runs. The relative centrifugal forces (*g*) were calculated for the center of the tubes.

60 gm of homogenate (7.2 gm of liver) were weighed into the tubes and centrifuged for 5 minutes at  $600 \times g$  to separate the nuclear fraction. To avoid disturbing the sediment the supernatant fluid was removed from this and the other fractions with a capillary pipette having an opening in the side of the sealed tip. The nuclei were resuspended in 8 ml of the sucrose medium and recentrifuged. The combined washings and supernatant solution were again centrifuged to remove most of the remaining nuclei. This small sediment was added to the nuclear fraction and the

nuclei were rewashed. The supernatant solution and washings were then centrifuged at  $19,000 \times g$  for 10 minutes to sediment the large granules. These particles were washed once by resuspending in 8 ml of sucrose solution and recentrifuging. The washings and supernatant fluid from the large granule fraction were centrifuged for 4 hours at  $19,000 \times g$ . After removing the final supernatant fluid as completely as possible the remaining sediment was taken as the small granule fraction. Each particulate fraction was evenly suspended in distilled water and appropriate aliquots were taken for analysis.

Analyses for pentosenucleic acid and desoxypentosenucleic acid were made according to the procedure of Schneider (8). For the riboflavin analyses<sup>2</sup> *Lactobacillus casei* was grown on the medium of Roberts and Snell (9), growth was determined by titration of the acid formed in 66 hours. The protein was determined gravimetrically after precipitation from aliquots containing 80 to 300 mg of protein with trichloroacetic acid at a final concentration of 10 per cent. After washing once with 1 M (pH 5) acetate buffer and twice with ethanol, the precipitate was transferred quantitatively to hard filter paper and extracted in a Soxhlet apparatus with ethanol for 48 hours. The extracted protein was transferred to weighing bottles, dried over  $\text{CaCl}_2$  *in vacuo* to a powder, and then left over  $\text{H}_2\text{SO}_4$  *in vacuo* for 12 hours. The product consisted almost entirely of protein and nucleoprotein (1). The total protein-bound aminoazo dye in these powders was determined as described previously (1).

### Results

*Comparison of 0.88 M Sucrose and 0.14 M KCl Solutions As Fractionation Media*—Preliminary simultaneous fractionations were carried out on the pooled livers of two rats fed the basal diet low in riboflavin to compare the intracellular distribution of nucleic acids, protein, and riboflavin when 0.14 M KCl (isotonic) or 0.88 M sucrose solutions were used as the fractionation media. Both solutions were initially buffered at pH 7.6 with 0.01 M phosphate. With either medium the nuclear fraction contained all or nearly all of the desoxypentosenucleic acid, the large granules from the partition in sucrose solution carried about 5 per cent of the total desoxypentosenucleic acid and microscopically a few nuclei could be seen in this fraction. However, in spite of the similarity in their contents of desoxypentosenucleic acid, the nuclear fraction from the separation in KCl contained three times as much protein and pentosenucleic acid and eight times as much riboflavin as the corresponding sample from the fractionation in sucrose solution (Table I). Correspondingly, the large granule fraction

<sup>2</sup> We are indebted to Mrs. G. M. Weber for these analyses.

obtained in the non-ionic medium contained two to three times as much of each of these constituents as when the separation was made in KCl solution. The composition of the small granule and supernatant fractions was less affected by the medium used for centrifugation, but the supernatant fluid from the fractionation in sucrose solution tended to contain more of each constituent than that from the centrifugations in KCl solution. The results are in agreement with the observations of Schneider (10) and Hogeboom *et al* (3, 4), who showed in other ways that the nuclei obtained from isotonic NaCl or KCl homogenates are grossly contaminated with agglutinated large granules. On the basis of these data 0.88 M sucrose solution was used as the medium for the subsequent fractionations, although even in this case the nuclear fraction is contaminated with a few whole cells and large granules.

TABLE I

*Comparison of Fractionation of Liver Homogenates in Either 0.88 M Sucrose or 0.14 M KCl*

Expressed as per cent of total component in the liver

Liver fraction	Pentose nucleic acid		Protein		Riboflavin	
	Fractionation medium					
	Sucrose	KCl	Sucrose	KCl	Sucrose	KCl
Nuclei	9	29	13	40	6	50
Large granules	33	17	30	11	64	23
Small “	26	31	12	8	9	8
Supernatant fluid	20	9	39	34	28	20
Recovery	88	86	95	94	107	101

*Protein Distribution*—The livers of rats fed *p*-dimethylaminoazobenzene for 4 weeks contained on the average only 119 mg of protein per gm of fresh weight as compared with 135 mg per gm for the livers of rats fed the basal diets (Table II). This decrease was almost entirely localized in the large granule fraction, since the protein content of these particles dropped on the average from 40 to 26 mg per gm of liver on feeding the dye. A 37 per cent increase in the protein of the nuclear fraction was also noted in rats fed dye in the low riboflavin diet. The recovery of protein (the sum of the fractions compared with the whole homogenate) varied from 93 to 103 per cent (average 96).

*Nucleic Acid Distribution*—Feeding *p*-dimethylaminoazobenzene to rats in the low riboflavin diet increased the amount of desoxypentose nucleic acid in the nuclear fraction by about 29 per cent (Table III). However, since the protein content of this fraction increased proportionately, the

TABLE II

*Distribution of Protein in Liver Fractions*

Mg of protein per gm of fresh liver, the figures to the nearest whole number

Liver fraction	Basal				Basal + dye			
	Low riboflavin		High riboflavin		Low riboflavin		High riboflavin	
	Fractionation N6							
	1	2	3	4	5	6	7	8
Whole homogenate	144	140	133	125	127	119	118	114
Nuclei	18	20	17	17	25	27	20	14
Large granules	42	41	40	38	29	26	23	27
Small “	19	17	16	16	17	16	16	13
Supernatant fluid	61	55	52	50	52	53	51	53
Recovery	140	133	125	121	123	122	110	107

TABLE III

*Distribution of Desoxypentosenucleic Acid and Pentosenucleic Acid in Liver Fractions*

Mg of nucleic acid per gm of fresh liver

Liver fraction	Basal				Basal + dye			
	Low riboflavin		High riboflavin		Low riboflavin		High riboflavin	
	Fractionation No							
	1	2	3	4	5	6	7	8
Desoxypentosenucleic acid								
Whole homogenate	2 52	2 49	2 34	2 27	3 41	3 04	2 13	2 03
Nuclei	2 28	2 41	2 28	2 20	3 21	2 85	1 98	1 94
Large granules	0 21	0 08	0 15	0 06	0 11	0 11	0 08	0 12
Small       “								
Supernatant fluid								
Recovery	2 49	2 49	2 43	2 26	3 32	2 96	2 06	2 06
Pentosenucleic acid								
Whole homogenate	7 06	5 83	6 83	6 05	5 00	4 63	4 81	4 16
Nuclei	0 47	0 60	0 61	0 58	0 74	0 66	0 64	0 34
Large granules	1 93	1 83	2 29	1 77	1 24	0 95	1 02	1 05
Small       “	1 99	1 68	1 79	1 66	1 33	1 02	1 18	0 95
Supernatant fluid	1 89	1 39	1 42	1 50	1 56	1 57	1 36	1 55
Recovery	6 28	5 50	6 11	5 51	4 87	4 20	4 20	3 89

ratio of desoxypentosenucleic acid to nuclear protein was similar for all four groups 91 to 98 per cent (average 95) of the desoxypentosenucleic acid was found in the nuclear fraction and 3 to 8 per cent (average 5) was in the large granules,<sup>3</sup> none was detected in the other fractions

The livers of animals fed the basal diets contained an average of 6.44 mg of pentosenucleic acid per gm, whereas the livers of animals receiving *p*-dimethylaminoazobenzene averaged 4.65 mg per gm (Table III). Among the fractions the largest reduction in pentosenucleic acid occurred in the large and small granules, the decreases were 45 and 37 per cent respectively. The large granules of livers from rats fed the basal diets contained an average of 49 mg of pentosenucleic acid per gm of protein, while the corresponding fraction from animals receiving the dye averaged 41 mg per gm of protein. The levels of pentosenucleic acid in the small granules were 105 and 74 mg per gm of protein for the livers of rats fed the basal and dye-containing diets, respectively. The greatest decrease in the ratio of pentosenucleic acid to protein was therefore in the small granule fraction. The contents of this acid in the nuclei and supernatant fluids did not differ significantly from group to group. Recoveries of this constituent ranged from 87 to 97 per cent (average 92).<sup>4</sup>

*Riboflavin Distribution*—Consumption of *p*-dimethylaminoazobenzene by rats receiving diets low or high in riboflavin reduced the level of riboflavin in their livers by 28 and 43 per cent respectively (Table IV). These effects were largely the result of decreases of 40 per cent in the level of this vitamin in the large granules and supernatant fluids of rats fed the low riboflavin diet and 50 per cent for those fed the high riboflavin diet. In contrast to these selective decreases the difference in riboflavin content of the livers from rats fed the basal diet low or high in riboflavin was due to a drop of 33 to 50 per cent in the vitamin content of each of the four fractions. In all cases the differences far exceeded any observed changes in protein content. The recoveries of riboflavin ranged from 94 to 104 per cent (average 101).

*Distribution of Protein-Bound Aminoazo Dyes*—The analyses for total protein-bound aminoazo dyes (Table V) are expressed arbitrarily as  $\log I_0/I \times 10^3$  per gm of liver, since the light absorption per unit weight of the dye released from the liver protein is not known (1). However, acid solutions of these dyes followed Beer's law, hence, the optical density was directly proportional to the concentration of the aminoazo dyes. The

<sup>3</sup> The desoxypentosenucleic acid is found exclusively in the nuclear fraction if the centrifugation time at 600*g* is increased to 10 minutes.

<sup>4</sup> Subsequent work has demonstrated that recoveries of pentosenucleic acid ranging from 93 to 99 per cent are obtained if the homogenates are kept at 0–3° up to the time the trichloroacetic acid is added.

livers were taken for analysis after 4 weeks of dye feeding, since the content of bound dye in the liver reaches a maximum at about this time. In agree-

TABLE IV  
*Distribution of Riboflavin in Liver Fractions*

Micrograms of riboflavin per gm. of fresh liver

Liver fraction	Basal				Basal + dye			
	Low riboflavin		High riboflavin		Low riboflavin		High riboflavin	
	Fractionation No							
	1	2	3	4	5	6	7	8
Whole homogenate	12.4	11.1	20.7	23.1	8.4	8.5	13.4	11.7
Nuclei	0.8	1.0	1.4	1.2	1.0	1.7	2.4	1.0
Large granules	6.2	5.3	11.0	12.3	3.3	3.7	5.6	5.9
Small “	1.6	1.2	2.6	2.8	2.6	1.0	1.9	2.3
Supernatant fluid	4.1	2.9	6.0	6.7	1.8	2.4	4.0	2.1
Recovery	12.7	10.4	21.0	23.0	8.7	8.8	13.9	11.3

TABLE V

*Distribution of Protein Bound Aminoazo Dyes in Liver Fractions of Rats Fed p-Dimethylaminoazobenzene\**

Measured in acid-ethanol at 520 m $\mu$  and expressed as  $\log I_0/I \times 10^3$  per gm. of fresh liver

Liver fraction	Low riboflavin		High riboflavin	
	Fractionation No			
	5	6	7	8
Whole homogenate	465	435	297	415
Nuclei	63	59	43	24
Large granules	72	50	35	49
Small “	58	69	42	47
Supernatant fluid	248	230	173	269
Recovery	441	408	293	389

\* The non specific absorption in the bound dye extracts was determined by carrying each of the protein samples from the liver fractions of the rats fed the basal diets (Fractionations 1 to 4) through the bound dye determination. The absorption of these extracts at 520 m $\mu$  gave the following corrections to be applied as  $\log I_0/I \times 10^3$  per 100 mg. of protein: whole 38, nuclei 68, large granules 42, small granules 46, and supernatant fluid 38.

ment with earlier work (1) feeding a high level of riboflavin with *p*-dimethylaminoazobenzene reduced the level of protein-bound dye in the liver. The



level of dye found in Fractionations 5, 6, and 7 was more nearly typical of other results than that for Fractionation 8, however, occasionally the ranges of dye content for animals fed the diet high and low in riboflavin overlap slightly when analyses are made at 4 to 6 weeks. In both cases the livers from animals fed the high level of riboflavin contained 29 to 44 per cent less protein-bound dye in the sedimentable fractions than the livers from rats on the low riboflavin diet, although the supernatant fluid from Fractionation 8 (high riboflavin) contained more bound dye than either supernatant fluid in the low riboflavin group. That all proteins do not carry the same amount of dye per unit weight is evident from a comparison of the levels of bound dye and protein (Table II) in the various fractions. For instance, the supernatant fluids, which contained an average of 44 per cent of the liver protein of dye-fed animals, had 57 per cent of the protein-bound dye, while the large granules with an average of 23 per cent of the liver protein contained only 13 per cent of the bound dye. The concentration of protein-bound dye in each fraction may be compared by calculating the optical densities per gm of protein. For Fractionations 5 and 6 the average values were whole homogenate 3.63, nuclei 2.37, large granules 2.20, small granules 4.22, and supernatant fluid 4.97. The recoveries of protein-bound aminoazo dye were from 94 to 99 per cent (average 96).

#### DISCUSSION

The selective decrease in the protein content of the small granule fraction of the rat liver following administration of *p*-dimethylaminoazobenzene is of interest since Schneider (11) found an even larger decrease in the protein content of the same fraction in hepatic neoplasms induced by this dye. Opie (12) noted that after feeding the carcinogen to rats for 1 or 2 months there was a histologically demonstrable decrease in the content of pentose-nucleic acid in the particulate elements of the liver cytoplasm. This is confirmed by the finding that a considerable reduction in the level of this constituent occurred in the isolated large and small granules from livers of rats treated in the same manner. It is established that the feeding of *p*-dimethylaminoazobenzene results in a large decrease in hepatic riboflavin (6, 13, 14). The loss of this vitamin has now been localized in the large granules and supernatant fluid. The highest concentrations of protein-bound dye occur in the small granules and supernatant fluid, but high levels of dietary riboflavin reduced the amount of bound dye in each of the four fractions to about the same extent. While the bound dyes appear to be involved in the carcinogenic process induced by the ingested dye (1), it is not yet possible to decide in which fraction, if any, they are of the most importance.

## SUMMARY

1 The livers of rats fed various diets for 4 weeks were homogenized and differentially centrifuged in a hypertonic sucrose medium to yield the nuclei, large granules, small granules, and a final supernatant fluid. The homogenates and fractions were analyzed for protein, nucleic acids, riboflavin, and protein-bound aminoazo dye.

2 The ingestion of *p*-dimethylaminoazobenzene in the diet low in riboflavin increased the protein and desoxypentosenucleic acid contents of the nuclear fraction by 37 and 29 per cent, respectively. Ingestion of the carcinogen in diets containing either low or high levels of riboflavin reduced the protein content of the large granules by 35 per cent, lowered the levels of pentosenucleic acid in the large and small granules by about 40 per cent, and decreased the levels of riboflavin in the large granules and supernatant fluid by 45 per cent.

3 When *p*-dimethylaminoazobenzene was fed, each liver fraction contained protein-bound aminoazo dye. The highest concentrations were found in the small granules and supernatant fluid. Ingestion of a high level of riboflavin lowered the level of bound dye in each fraction.

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# THE DISTRIBUTION PATTERN OF SULFUR-LABELED METHIONINE IN THE PROTEIN AND THE FREE AMINO ACID FRACTION OF TISSUES AFTER INTRAVENOUS ADMINISTRATION\*

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As a result of studies with isotopically labeled amino acids in recent years, it has become firmly established that there is a continuous interchange between the amino acids of body protein and free amino acids supplied by the circulation. In most of the previous experiments on the incorporation of labeled amino acids into protein, the animals were sacrificed at a single time interval after administration of the amino acid. Few experiments on the change in labeled amino acid uptake with time have been reported. Furthermore, in practically all instances the labeled amino acids have been administered orally. This exposes the intestinal mucosa and liver to much higher concentrations of amino acids initially than the other tissues and may yield a false picture of the synthetic activities of these tissues. In the experiments to be reported here, the labeled amino acid has been injected intravenously into rats.

Other experiments have also demonstrated marked differences in the rate of incorporation of labeled amino acids by various tissues and organs, intestinal mucosa, kidney, and liver exhibiting a high rate, and muscle and brain a very much lower rate. Since it has already been shown that the intestinal mucosa is more active in the incorporation of amino acid than any other tissue (1, 2), it is important to see whether the activity varies along its length as does the function. In order to study this, additional experiments have been performed on dogs, which were likewise injected intravenously with methionine.

## EXPERIMENTAL

### *Experiments with Rats*

**Protein Fraction**—DL-Methionine containing S<sup>35</sup> (3, 1) was added to an amino acid hydrolysate (parenamine, Stearns) and injected by way of the jugular vein into male rats weighing about 150 gm. In the experiments

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† National Cancer Institute Senior Research Fellow, 1947-48.

presented in Fig 1, the rats were fasted 4 hours before the injection of 0.8 ml of the parenamine-methionine mixture,<sup>1</sup> and no food was given thereafter. In the experiments summarized in Fig 2, the rats were fasted 6 hours before the injection of 2 ml of the parenamine-methionine mixture, and then they were immediately placed on a regular stock diet.

At intervals as indicated in Figs 1 and 2 an animal was sacrificed, the protein was precipitated from the homogenized tissues with 10 per cent trichloroacetic acid and washed four times with 5 per cent trichloroacetic acid. The product was digested with Pirie's reagent and analyzed for inorganic sulfur by benzidine precipitation and subsequent titration with 0.05 N NaOH, and for radioactive sulfur by barium chloride precipitation and counting in the Geiger-Müller counter (4).

*Free Amino Acid Fraction*—Two male rats (about 120 gm each) received labeled methionine equivalent to 0.04 gm of amino nitrogen per kilo of body weight by way of the jugular vein. 15 minutes after the injection the blood plasma and the desired tissues were collected and 5 per cent trichloroacetic acid filtrates were prepared by adding 5 ml of the acid to about 1 gm of tissue (accurately weighed) in a glass homogenizer. An aliquot of the filtrate was digested with Pirie's reagent and after acidification the sulfur was precipitated with 10 per cent barium chloride and the radioactivity determined. To obtain a sizable sulfate precipitate, 10 ml of 0.05 N H<sub>2</sub>SO<sub>4</sub> were added to all samples.

#### *Experiments with Dogs*

Two dogs (weighing 1.6 and 2.3 kilos) were injected intravenously (femoral vein) with 1 and 2 ml, respectively, of 0.9 per cent sodium chloride containing a trace of S<sup>35</sup>-labeled methionine. Food was removed 6 hours prior to the injection, and 6 hours after the injection the animals were sacrificed. The entire gastrointestinal tract was removed and flushed with water, the mucosa (and submucosa) was obtained by pressure from the outside of the tract, and the activity of the proteins, precipitated with 10 per cent trichloroacetic acid, was determined.

#### DISCUSSION

The present experiments demonstrate that the *pattern* of distribution of the labeled amino acid is essentially the same regardless of whether the amino acid is administered orally or intravenously, in trace amounts or in considerable quantity. However, it should be noted that the actual uptake by the tissues is less in these experiments than in those in which the methionine was fed in small doses (5).

<sup>1</sup> This mixture consists of a solution of parenamine (a protein hydrolysate) with a content of 16 mg of total  $\alpha$ -amino nitrogen and 36 mg of methionine per ml. A small amount of labeled methionine (about 0.1 mg possessing 16,000 counts per minute) was added to each ml.

In the fasted animals (Fig 1) a period of rapid incorporation of the amino acid into the protein is followed by a period of stabilization of the tagged sulfur within the various organs. *The intestinal mucosa proteins show the highest specific activity, but they also exhibit a rapid loss of activity, i e a rapid turnover.* The support of the high rate of secretion of protein into the intestine as enzymes and as mucous proteins requires a high rate of protein synthesis (2). Other organs (*e g*, the pancreas) that secrete proteins in large amounts would also be expected to show rapid incorporation

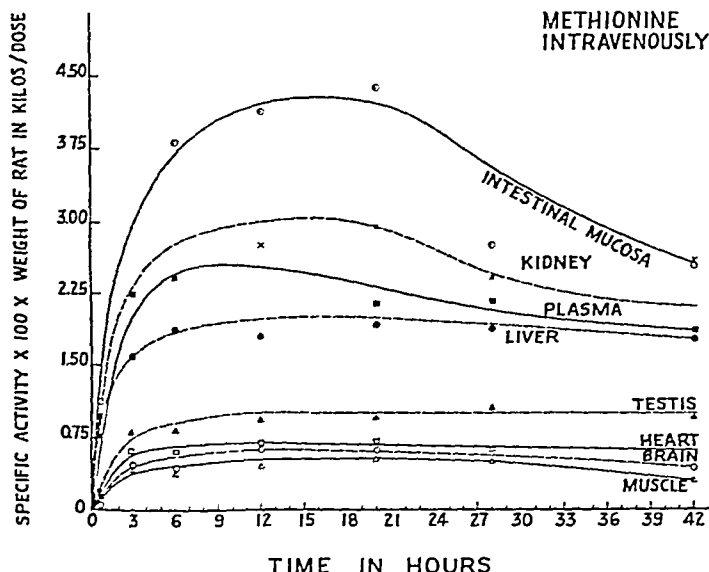


FIG 1 Specific activity of sulfur in the protein of rat tissues immediately after intravenous injection of labeled methionine. The rising parts of the curves illustrate the incorporation of the sulfur-containing amino acids into the tissue proteins.

of methionine. Since the pancreas of the rat is diffuse and not easily removed as a unit, a separate experiment on dogs was performed. 6 hours after the intravenous administration of labeled methionine to dogs, the following pattern of activity was obtained: muscle 0.04,<sup>2</sup> thymus 0.19, liver 0.23, lung 0.24, kidney 0.37, spleen 0.38, pancreas 0.39, and intestinal mucosa 0.80. *The value for the pancreas, therefore, is second only to that of the intestinal mucosa.*

The pattern of activity shortly ( $\frac{1}{2}$  hour) after intravenous administration of the amino acid is not identical with that observed after a longer time interval (3 to 42 hours) (see Fig 1). In the first half hour the specific

<sup>2</sup> Expressed as (specific activity  $\times$  100  $\times$  weight of animal in kilos)/(dose administered).

activity of the plasma is remarkably low (the values of the proteins of the liver are higher than those of the proteins of the kidney or plasma), and the proteins of the intestinal mucosa already show the highest activity. Repetition of this experiment gave identical results. Several hours later, the following pattern in order of decreasing activity exists: intestinal mucosa, kidney, plasma, liver, testis, heart, brain, and muscle. The belated activity of the plasma protein is not surprising, since in all probability these proteins are formed mainly in the liver and poured into the plasma (6). The finding that the specific activity of liver protein is lower than the specific

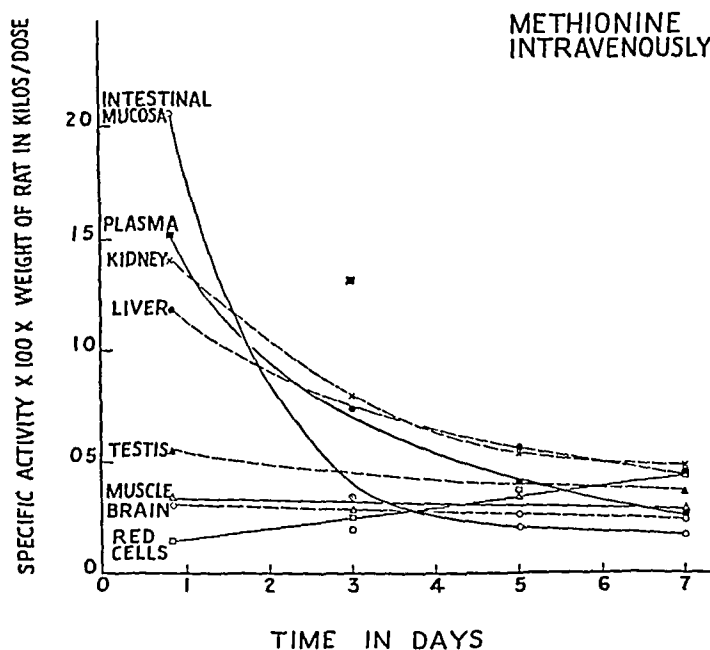


FIG 2 Specific activity of sulfur in the proteins of rat tissues in the later stages after intravenous injection of labeled methionine. The curves in this figure illustrate the rate of dilution of radioactive methionine by inert sulfur.

activity of plasma protein (except within the first half hour) at first sight is difficult to reconcile with the general concept that the liver is the primary site of plasma production. A plausible explanation is that the liver value represents an average of secretory proteins of high specific activity and structural proteins of low specific activity within the same organ.

The pattern of distribution described here with methionine is very similar to that obtained by the use of carbon-labeled tyrosine (7) and thus suggests that the same metabolic process is responsible in both cases.

The value for the protein of red blood cells, which at first is lower than for that of any other organ, rises continuously, while the proteins of all other tissues and that of the plasma exhibit a downward trend (Fig 2).

Hence, the curves for the proteins of plasma and red cells intersect between the 5th and 6th day after the administration of the labeled amino acid. Probably this is due to the time required for the maturation of the red cell even after its proteins have incorporated the labeled methionine.

The activity levels of  $S^{35}$  in the proteins of the various organs tend to approach a common value. *This tendency for equipartition is slow as compared to the rapid rate of incorporation.* The proteins of the more active tissues (kidney, liver) show a faster decline in their specific activity than do those of the inert tissues (muscle, brain, etc.). It should be noted that the proteins of the plasma lose their activity faster than those of the liver, possibly because the plasma proteins serve not only for the maintenance

TABLE I

*Distribution of Labeled Sulfur in Free Amino Acid Fraction 15 Minutes after Administration of Labeled Methionine\**

Tissue	Rat 1	Rat 2
Kidney	1 53	2 74
Spleen	1 05	1 44
Plasma	0 96	1 34
Liver	0 88	1 22
Lung		1 08
Intestine		0 77
Intestinal mucosa	0 40	0 41
Muscle	0 34	0 65
Testis	0 32	0 34
Brain	0 12	0 18

\* Activities are expressed as follows (counts (per minute) per 100 gm of tissue or 100 ml of plasma)/(counts in administered dose (per minute))

of the vascular osmotic pressure, but also as a source of protein material for other tissues of the body.

From the distribution of  $S^{35}$  in the amino acid filtrates, determined 15 minutes after the injection of labeled methionine (Table I), it can be seen that *the pattern of concentration for the free amino acid is similar to that for sulfur incorporated into the protein.* However, the intestine no longer shows the highest degree of accumulation. This picture is not altered even if the results are expressed on the basis of *dry weight*. Furthermore, the level of  $S^{35}$  activity in the muscle is much higher than that of the brain, whereas the incorporation of  $S^{35}$  into the protein of these two tissues is nearly identical. The inability of the brain to concentrate amino acids has been reported previously (8). If an amino acid is injected into the cisterna magna of an animal, however, the blood-brain barrier is by-passed



and the protein of liver, plasma, or kidney will incorporate less amino acid than the protein of the brain<sup>3</sup>

The rapid rate of incorporation and removal of methionine from the protein of the intestinal mucosa<sup>4</sup> is shown in Figs 1 and 2. The variation in incorporation along the length of the gastrointestinal tract in the two dogs is shown in Fig 3. The incorporation in the stomach mucosa was very low, and three peaks of activity were found in the intestinal mucosa. These peaks appeared in the region of the junction of the duodenum and jejunum, the junction of the jejunum and ileum, and in the terminal segment of the large intestine.

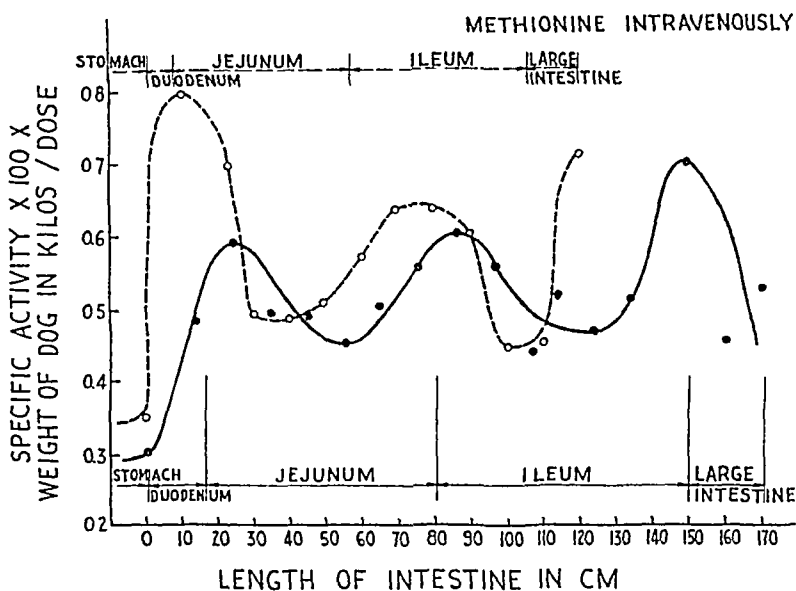


FIG 3 Specific activity of sulfur in the protein of the mucosa of different parts of the gastrointestinal tract after intravenous injection of labeled methionine. The upper labels of the gastrointestinal tract refer to the dashed curve, the lower labels to the solid curve.

#### SUMMARY

The distribution pattern of the labeled sulfur in the proteins and the free amino acid fraction of the tissues of fasted rats after the intravenous administration of methionine labeled with radioactive sulfur has been determined.

A period of rapid incorporation of the amino acid into the protein is

<sup>3</sup> Friedberg, F, and Greenberg, D M, unpublished data.

<sup>4</sup> It is intestinal mucosa that exhibits a high specific activity and not intestinal muscle. The latter shows a slightly lower value than does the liver. The following is an illustrative example: activities at 6 hours after administration, intestinal muscle 0.66, intestinal mucosa 1.21, liver 0.76, kidney 1.07.

followed by stabilization of the tagged sulfur within the various organs. The pattern of incorporation of  $S^{35}$  in order of decreasing activity was as follows: intestinal mucosa, pancreas, spleen, kidney, plasma, liver, testis, heart, brain, and muscle. The value for the protein of red blood cells, which at first is lower than that of any other organ, rises continuously, while all tissues and the plasma exhibit a downward trend.

In the dog the rate of incorporation is very low for the proteins of the stomach mucosa. Maxima of uptake were observed in the region of the junction of the duodenum and jejunum, the junction of the jejunum and ileum, and in the terminal segment of the large intestine.

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# A SIMULTANEOUS CARR-PRICE REACTION FOR THE DETERMINATION OF VITAMIN A

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There are several shortcomings in the estimation of vitamin A from the color developed by the Carr-Price reagent. Most of the interfering substances can be removed by saponification, but development and fading of the blue color depend also on the age of the reagent (1) and the intensity of light to which the mixture is exposed (2). Increased temperature seems to affect the stability of the color (3), and even different batches of  $\text{SbCl}_3$  give discordant results (4).

Earlier attempts to match the color in a block comparator with solutions of copper and cobalt salts (5) were therefore superseded by methods with direct reading photoelectric colorimeters for estimating the fleeting color, the results (6), however, with different instruments vary.

An attempt has therefore been made to work out a method in which unknown and standard vitamin A solutions are treated simultaneously. This procedure avoids most of the above pitfalls.

## Principle

A vitamin A ester distillate of known strength serves as a standard. From this vitamin A ester two dilutions with chloroform are prepared, 1:5. By comparing the Carr-Price reaction in both of these dilutions of the standard a calibration diagram is obtained. Then the strength of the unknown is compared with that of the weaker standard, and its vitamin A content read from the calibration diagram.

### Materials—

Chloroform

Saturated solution of  $\text{SbCl}_3$  in chloroform

Acetic acid (concentrated)

Distilled vitamin A concentrate (containing 201,500 U. S. P. units per gm., or 5.65 per cent of vitamin A<sup>1</sup>)

## Apparatus and Methods

**Colorimeter**—A Lumetron colorimeter is modified for this purpose (Fig. 1). The light from a tungsten lamp (1) passes a lens (2), a Corning filter

<sup>1</sup> We are indebted to Distillation Products, Inc., Rochester 13, New York, for their kind gift of standardized vitamin A capsules.

(3) with a peak at  $610\text{ m}\mu$ ,<sup>2</sup> two test-tubes (4 and 4'), and strikes two similar photocells (5 and 5') of the barrier layer type. Both cells can be turned around the vertical axis (as indicated by curved arrows) which changes the angle of incidence and thus the amount of light reaching them. The electromotive forces developed by the cells are compared on a calibrated bridge (6) which reads from 0 to 100 per cent transmission, zero being indicated by a sensitive galvanometer (7).

*Double Pipette*—Two 5 ml graduated pipettes (to deliver) are selected which are equal in length when measured from the tip to the 4 ml mark.

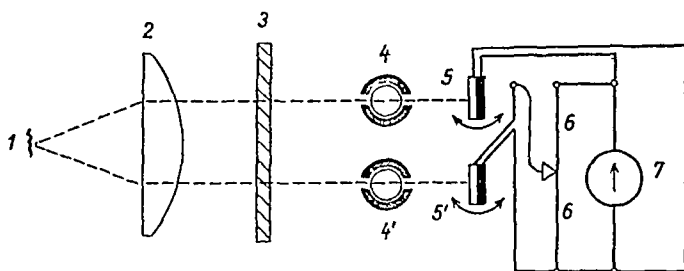


FIG 1 Diagram of modified Lumetron colorimeter. Light from a lamp (1) passes a lens (2), a filter ( $610\text{ m}\mu$ ) (3), two test-tubes (4, 4'), and strikes two photocells (5, 5') which can be tilted as indicated by the arrows. The current generated by both cells is balanced on a slide wire bridge (6), the galvanometer (7) indicating zero. While 1, 2, 3, 6, and 7 are standard equipment of the Lumetron, the test-tube holders (4, 4') and the photocells (5 and 5') are attached to an ebonite plate similar to the test-tube adapter of the Lumetron. 5 and 5' are rectangular photocells ( $16 \times 36\text{ mm}$ ) which are available as spares for light meters. The cells are suspended from a vertical shaft which passes through the ebonite cover in a light-tight packing. An adjusting knob attached to the upper (outer) end of the shaft of each photocell facilitates tilting.

They are cut off slightly above the 5 ml mark and joined, parallel to each other, by a T-shaped glass tube so that the double pipette looks like an inverted Warburg manometer. The length of both legs should equal that of the test-tubes in the colorimeter (Fig 1, 4, 4'). As both pipettes are to deliver an equal amount of the Carr-Price reagent simultaneously and with equal speed, their time of delivery should be checked with water or glycerol. If there is any difference, the tip of the slower one is widened by grinding.

### Procedure

Several dilutions of the standard in chloroform are prepared, containing 10, 20, 25, 30, 40, and 50 U S P units of vitamin A per ml respectively.

<sup>2</sup> Filter 620 would be more specific, but Filter 610 was chosen as it has a greater over-all transmission and thus allows rather concentrated solutions to be tested.

Two matched test-tubes, filled with chloroform, are inserted into the colorimeter (Fig 1, 4, 4') The bridge (6) is set to read 80 per cent transmission, this value is arbitrary but proves to be convenient Then the resistance of the light source (1) is adjusted to yield a convenient intensity

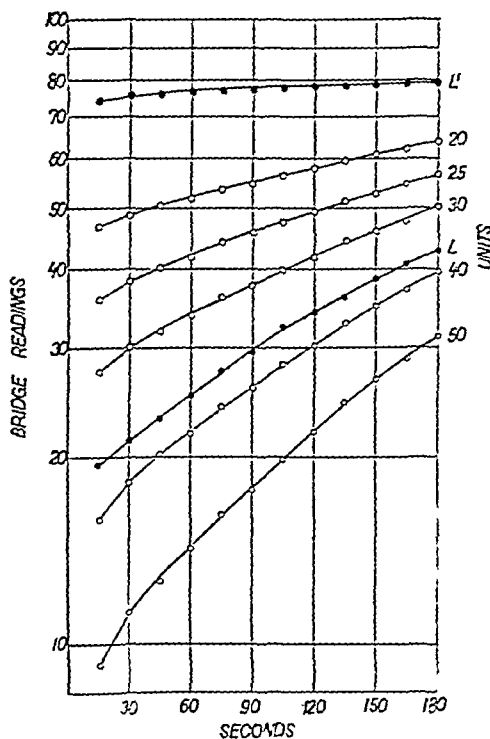


FIG 2 Difference in fading of Carr-Price color The bridge shown in Fig 1 is set to read 80 when starting Different concentrations of the standard (O), viz 50, 40, 30, 25, and 20 U S P units per ml, are compared with a standard containing 10 U S P units per ml Note that with falling concentration the curves become flatter, change their slope, and approach the 80 bridge reading line  $L$  and  $L'$  (●) are two dilutions of the non-saponifiable fraction of sheep liver

of light Light intensity is estimated by connecting the photocell (5) directly to the galvanometer (7) Though strict maintenance of the light intensity is not necessary, as a balanced circuit is used (Fig 1), reproducibility is better if the light intensity is similar in subsequent tests By slightly tilting one or both of the photocells a zero reading on the galvanometer (7) is obtained The test-tubes containing chloroform are now replaced by two others to which 1 ml of two of the standard dilutions has

been transferred, *e g*, 1 ml of the 10 unit and 1 ml of the 50 unit standard respectively. As can be deduced from the wiring diagram in Fig 1, the stronger standard has to be inserted into the test-tube (4). About 3 drops of acetic acid are now added to either test-tube. The Cari-Price reagent is drawn into the double pipette up to the 4 ml mark from a wide mouthed

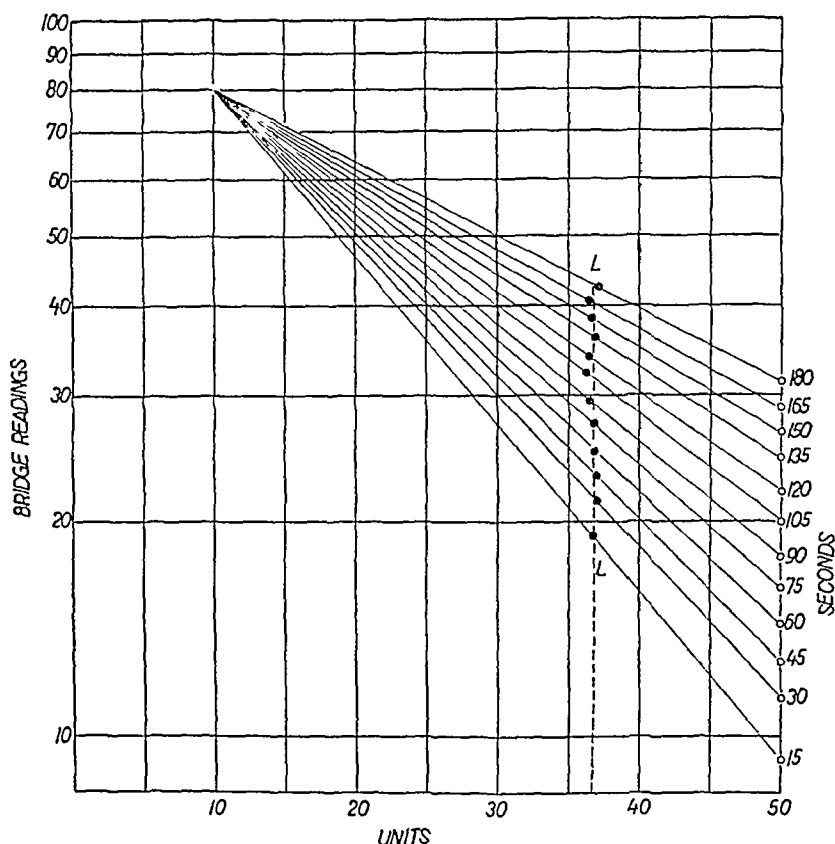


FIG 3 Calibration diagram for determining unknown concentrations. This figure is complementary to Fig 2. The 50 U S P unit bridge readings (line next to the bottom in Fig 2) are plotted on the 50 unit ordinate in Fig 3. All of these points are connected by straight lines with a point where the 10 unit line (abscissa) and the 80 bridge reading line (ordinate) meet. Unknown concentrations are determined simply by entering each bridge reading on the appropriate seconds line (●, *L-L*), and plotting the best fitting straight line (broken line) back to the abscissa, where the result (36.7 units) is read. The dots are identical with line *L* in Fig 2.

bottle and delivered into both test-tubes containing the standard. As soon as delivery of the reagent commences, a stop-watch is started, and readings on the measuring bridge (Fig 1, 6) are taken every 15 seconds. The curve marked 50 on the right-hand scale of Fig 2 shows bridge readings *versus* time for 50 U S P units when compared simultaneously with 10 U S P

units When the test is repeated with 40 U S P units and 10 U S P units as the weaker standard, the curve marked 40 on the right-hand in Fig 2 is obtained Again, if 40 U S P units are replaced by 30, 25, and 20 U S P units respectively and the weaker standard kept at 10 U S P units, a family of curves, indicated in Fig 2 by hollow circles, emerges An unknown substance may be interpolated by comparing it similarly with the standard containing 10 U S P units per ml Curves *L* and *L'* (Fig 2) show two dilutions of the unknown, the non-saponifiable fraction of sheep liver It can be readily seen that determining the unknown by interpolation is rather cumbersome, and a simpler method, described below, is therefore advocated

### Calculations

By plotting bridge readings *versus* concentration of vitamin A, and connecting the points of equal time, straight lines are obtained (Fig 3) which converge to a point situated where the 10 unit line (abscissa) meets the 80 per cent bridge reading line (ordinate) The common intersection point simply reflects the fact that the galvanometer remains stationary (indicating zero) at all times if both test-tubes contain equal amounts of vitamin A. As the lines obtained in Fig 3 are straight and their intersection is known in advance, all one has to do is to prepare two standard dilutions It will be readily appreciated that an unknown is more easily determined from Fig 3 than from Fig 2 Line *L* in Fig 3 is identical with line *L* in Fig 2 To estimate an unknown from Fig 3 all that has to be done is to connect the dots by the best fitting straight line and plot it back to the abscissa, giving 36.7 U S P units per ml as an estimate of the unknown

### DISCUSSION

It has been pointed out (2) that low intensities of light should be used in measuring the Carr-Price color, as it is not stable towards light, in the proposed method standard and unknown are exposed to the same intensity of light, and only the difference in fading between standard and unknown is measured Though the unknown should fall between two concentrations of the standard, a fair estimate may still be obtained if the dilution of the unknown contains less vitamin A than the weaker standard, for the guiding lines shown in Fig 3 still hold good when extended beyond the common intersection point But as readings towards the upper end of the scale become less reliable, a result obtained above 90 per cent on the scale reading would indicate that a more appropriate dilution should be chosen for the next experiment Though the Carr-Price color fades, this does not seem to interfere with the reliability of readings of the unknown obtained at different times after the reagent is added, for the cluster of dots (repre-



senting the unknown in Fig 3) seems randomized To test this point further let us compare two dilutions of the unknown, *viz*  $L$  and  $L'$  (see Fig 2), where  $L'$  has been obtained by adding 7 ml of chloroform to 3 ml of  $L$  The estimate of  $L'$  is performed from Fig 3, as was done for  $L$  As already mentioned, the estimate for dilution  $L$  is 36.7 units (mean), with a standard deviation of 0.27 unit,  $L'$  yields 11.2 units (mean), with a standard deviation of 0.21 unit This seems to be in favor of the higher dilution ( $L'$ ), as its standard deviation is smaller, but this advantage is offset, as the coefficient of variation is

$$\frac{100 \times 0.27}{36.7} = 0.74 \text{ for } L, \text{ but } \frac{100 \times 0.21}{11.2} = 1.87 \text{ for } L'$$

It does not therefore seem that accuracy would be greatly enhanced by making the standard and unknown nearly identical It will, however, be noticed that both dilutions yield practically the same estimate

The result obtained from the Carr-Price reaction is also in fair agreement with the figure obtained from the absorption in the ultraviolet For determining the ultraviolet absorption the unknown and standard are diluted to read 50 per cent transmission, with the blank as 100 per cent transmission (7), this value rather than  $E_{1\text{cm}}^{1\%}$  is used in estimating the unknown<sup>3</sup> We thus find 492.5 U. S. P. units per gm. of sheep liver from the ultraviolet absorption and 443 U. S. P. units per gm. from the pooled estimates ( $L'$  and  $L$ ) shown in Fig. 2

The method as described above is applicable to the estimation of vitamin in liver and oils Experiments are under way to adapt the simultaneous Carr-Price reaction to materials low in vitamin A and to those cases as well in which interfering matter cannot readily be eliminated by saponification

#### SUMMARY

A method is described for determining vitamin A simultaneously in standard and unknown, thus canceling errors due to different treatment of standard and sample Readings of the Carr-Price color can be taken more at leisure, as the difference in fading rather than the fading of the color itself is measured A simple reference diagram for converting readings into vitamin A units is given

<sup>3</sup> As  $E_{1\text{cm}}^{1\%}$  cannot be actually measured in determining the ultraviolet absorption of vitamin A, the density being too high, the half value,  $W_{1\text{cm}}^{50}$ , is suggested instead (7) The half value is the weight of a substance (in gm.) which has to be contained in 1 ml. of solvent in order to reduce the transmitted light by half, the length of the path being 1 cm. and the light transmitted by the solvent alone being unity By falling always into the sensitive range,  $W_{1\text{cm}}^{50}$  does not call for extrapolation as in the case of  $E_{1\text{cm}}^{1\%}$  Obviously, when a solution follows Beer's law over a very wide range  $E_{1\text{cm}}^{1\%}$  and  $W_{1\text{cm}}^{50}$  are connected by  $E_{1\text{cm}}^{1\%} = 0.00301/W_{1\text{cm}}^{50}$

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# TECHNIQUES FOR IN VIVO TRACER STUDIES WITH RADIOACTIVE CARBON\*

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In view of the increasing importance of radioactive  $C^{14}$  in biological studies and the lack of extensive literature on methods for assaying this isotope in animal tissues and fluids, it is felt that such methods as have been found useful might be worthy of recording. Described herein are techniques for the collection of excretory products suspected of containing isotopic carbon from mice, quantitative oxidation of tissues and excretory products prior to activity assay, and an application of the method of Miller (1) with slight variations for assay of  $BaCO_3$  resulting from the above oxidation.

## EXPERIMENTAL

*Metabolism Chamber for Collection of Excretory Products*—In an attempt to carry out a balanced experiment in tracer biochemistry with carbon isotopes, it is of course necessary to include the respiratory system as a possibly important excretory route. This fact, along with such others as economy of carbon isotope and dilution factors related to animal weight, may influence one to choose the mouse as an experimental animal, at least in exploratory studies.

In order to measure the quantity and rate of excretion of active carbon by the respiratory route, it is necessary to utilize an absorption line in which the  $CO_2$  exhaled by the animal can be quantitatively absorbed for subsequent precipitation and weighing prior to radioactive assay. A sketch of the apparatus employed in our laboratory for this purpose is presented as Fig. 1. By means of the legend on the sketch, the operation of the chamber may be described as follows:

Room air is drawn through a wet test meter, *A*, and successively through absorption bottles containing 10 per cent sodium hydroxide, *B*, saturated barium hydroxide, *C*, and saturated sodium chloride, *D*. The carbon dioxide is removed from room air in *B*, the barium hydroxide absorption bottle, *C*, is used simply as an indicator of the adequacy of *B*. The saturated sodium chloride solution, *D*, is employed for equilibration of humidity.

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in the mouse chamber, *E*, to approximately 75 per cent. Feces are collected on a wire screen, urine samples are collected in tube *F*. Water is supplied to the mouse from bottle *G*. The carbon dioxide exhaled by the test animal in the glass chamber *E* is absorbed in  $\text{CO}_2$ -free sodium hydroxide, *H*. Absorption bottle *I*, containing saturated barium hydroxide, is used as an absorption efficiency indicator. Air flow is maintained at a constant rate of about 300 ml per minute by means of a critical orifice, *J*, constructed of capillary tubing, one end of which has been constricted by fine polishing until the proper line air flow is obtained. A pressure drop across the orifice is maintained at greater than the critical pressure for air by means of a vacuum pump. This requires a pressure differential equal to or greater than 14.5 inches of mercury.

In view of the fact that the respiration of a 20 gm mouse is of the order of 22 ml per minute and the  $\text{CO}_2$  content of the expired air is approxi-

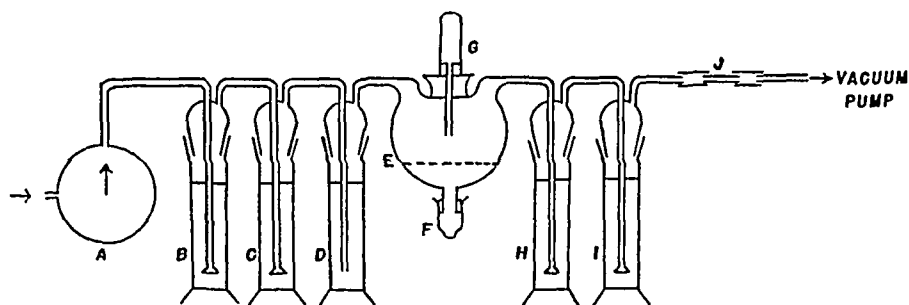


FIG 1 Apparatus for collecting respiratory carbon dioxide and other excretory products. *A*, wet test meter, *B*, *C*, and *D*, absorption bottles containing 10 per cent sodium hydroxide, saturated barium hydroxide, and saturated sodium chloride, *E*, mouse chamber, *F*, urine collection tube, *G*, water bottle, *H* and *I*, absorption bottles containing  $\text{CO}_2$ -free sodium hydroxide and saturated barium hydroxide, *J*, orifice

mately 4 per cent, it may be estimated that about 0.05 mole of carbon dioxide will be expired by a 20 gm mouse in 24 hours.

Typical data, obtained with the absorption line, to collect  $\text{CO}_2$  expired by a 25 gm mouse are given in Table I.

It is important to maintain an air flow in the mouse chamber great enough to prevent accumulation of  $\text{CO}_2$ , which might affect respiratory rate. Excessively high flow rates, however, result in incomplete absorption. When short collection periods were used, we have increased our chamber air flow for a few seconds at the end of each period so that the time for reaching theoretical clearance of the chamber is very short as calculated by Silver's equation  $t_x = K(a/b)$  where  $\tau$  is the per cent of nominal concentration attained in time  $t$  (minutes),  $K$  is a constant, depending on the desired degree of equilibration (4.6 for 99 per cent),  $a$  is the volume of the chamber, and  $b$  is the volume of air passing through the chamber each minute (2).

*Oxidation of Animal Tissues for Determination of Radioactive Carbon*—Because of the low energy radiation of  $C^{14}$  and absorption factors, it is impossible to carry out activity assays in tissues *per se*. This difficulty can be overcome by oxidizing the tissues to carbon dioxide and counting in this form or as barium carbonate. The procedure we have found satisfactory for the oxidation of tissues is an adaptation of the method of Van Slyke and Folch (3) for manometric carbon determination. The method depends on combustion in a mixture of chromic, iodic, sulfuric, and phosphoric acids, and the preparation of the reagents has been described by these authors. In the present work, the carbon dioxide is not determined manometrically but is absorbed in sodium hydroxide solution and precipitated as barium carbonate. The latter is weighed in order to calculate

TABLE I  
*Carbon Dioxide Output of 25 Gm. Mouse*

Absorption period	Barium carbonate recovered	
	Per period	Cumulative
<i>min</i>	<i>gm</i>	<i>gm</i>
0-10	0.1841	0.1841
10-20	0.1203	0.3044
20-30	0.1647	0.4691
30-60	0.4086	0.8777
<i>hrs</i>		
1-2	0.6700	1.5477
2-3	0.6783	2.2260
3-4	0.6597	2.8857
4-5	0.6724	3.5581
5-6	0.6243	4.1824
6-24	10.0979	14.2703

the total carbon content of the tissues and is then reconverted to carbon dioxide for determination of the specific activity in the manner described in another section of this report. The reasons for this precipitation of  $CO_2$  and subsequent regeneration for activity assay are that (a) it is convenient for different operators to carry out the oxidations and the activity assays, (b) it is desirable to save samples for future check and (c) it is not feasible to allow the acid vapors from the tissue oxidation to enter the Geiger tube. The apparatus for oxidation of tissues and absorption of  $CO_2$  is shown in Fig. 2. The procedure used is given as follows:

The sample and potassium iodate are placed in small flask *E*, carbon dioxide-free 10 per cent sodium hydroxide in absorption cell *B*, and barium chloride solution in tube *D*. The small tube *A* contains saturated barium hydroxide solution as an indicator for completeness of carbon dioxide

absorption While a slow stream of nitrogen (or carbon dioxide-free air) is passed through the system, the combustion fluid is added slowly from funnel *C* The flask is warmed gently and then gradually heated to boiling The carbon dioxide formed by the oxidation passes into the absorption cell, *B*, through the sintered glass plate, *P*, which breaks it into fine bubbles After the oxidation is complete, the apparatus is flushed with nitrogen and the sodium hydroxide is run through stop-cock *d* into tube *D* where the carbonate is precipitated as barium carbonate The walls of *B* are carefully washed with water Stop-cock *a* is opened to allow the washing of the inlet tube

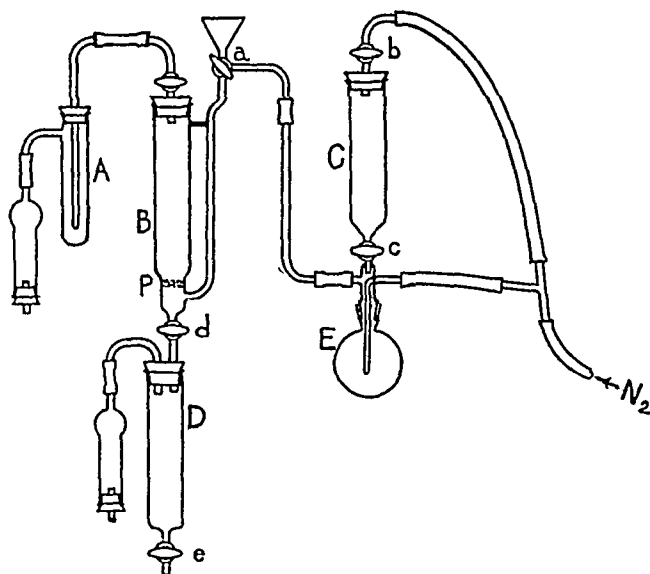


FIG 2 Apparatus for tissue oxidation *A*, tube containing saturated barium hydroxide, *B*, absorption cell containing  $\text{CO}_2$ -free 10 per cent sodium hydroxide, *C*, funnel, *D*, tube containing barium chloride solution, *E*, flask containing sample and potassium iodate, *P*, sintered glass plate, *a*, *b*, *c*, *d*, and *e*, stop cocks

The bottom of tube *D* is connected to the top of a small, tared, sintered glass funnel and the mixture in *D* filtered by suction through stop-cock *c* (which has a wide bore) with the careful exclusion of air Particles of the precipitate are washed from *D* onto the funnel and the latter is washed until free of alkali The funnel containing the barium carbonate is dried for a short time in an oven at  $110^\circ$  and then in a vacuum desiccator before weighing

The entire operation is carried out under a hood to avoid breathing any of the carbon dioxide which might possibly escape

Results from the oxidation of samples of known composition are recorded in Table II, while those from the tissue combustions are given in Table III

*Determination of Radioactive Carbon*—The specific activity of barium carbonate samples is determined by a slight modification of the method of Miller (1), in which the carbonate is converted to carbon dioxide and the latter introduced into the counter tube

The apparatus is diagrammed schematically in Fig 3. Essentially, the procedure involves the liberation of carbon dioxide from the barium carbonate by addition of perchloric acid, the removal of water by condensation at about  $-80^{\circ}$ , the collection of the carbon dioxide by condensation at about  $-195^{\circ}$ , the volumetric measurement of the quantity of carbon dioxide, the addition of a predetermined quantity of carbon disulfide, the condensa-

TABLE II  
*Analysis of Samples of Known Composition*

Sample	Weight of sample	Weight of BaCO <sub>3</sub>		Error	Combustion Time*
		Calculated	Found		
	gm	gm	gm	per cent	min
Benzoic acid	0.0294	0.3328	0.3320	0.24	10-12
	0.0268	0.3033	0.2987	1.50	10-12
	0.0250	0.2830	0.2801	1.02	10-12
Cystine	0.0255	0.1257	0.1238	1.51	8
	0.0271	0.1336	0.1361	1.87	8
	0.0248	0.3088	0.2950	4.46	5
Fatty acid†	0.0309	0.3844	0.3639	5.33	6
	0.0307	0.3828	0.3687	3.68	6
	0.0240	0.2991	0.3055	2.14	7
	0.0264	0.3329	0.3243	2.58	7
	0.0330	0.4113	0.4068	1.10	8
	0.0285	0.3552	0.3495	1.60	10

\* Period of heating

† Neo fat-1-65, Armour and Company, stearic acid 90 per cent, oleic acid 4 per cent, palmitic acid 6 per cent. Microanalysis showed 75.8 per cent carbon

tion of the resulting mixture in the Geiger-Muller tube, the thorough mixing of the gases after evaporation, and the measurement of the counting rate.

Certain details of the apparatus warrant further mention. The water reservoir, *Q*, for the carbon dioxide generator, *O*, is provided for ease in rinsing down the walls after each generation, if the water is drawn in while the generator is under reduced pressure, thorough rinsing occurs. The water trap, *N*, is of concentric tube design. Its outer jacket is removable at a ground glass joint for convenient drainage of collected water. The volume of the "doser" bulb, *K*, is such that, when it is filled with carbon disulfide vapor in equilibrium with the liquid at the ice point, it contains the proper amount to produce 2 cm. of carbon disulfide pressure in the counter



TABLE III

*Oxidation of Mouse Tissues*

Tissue A, normal mouse, 25 gm, Tissues B and C, mice, 25 gm each, injected with radioactive urethan (35 mg, 2.49 microcuries)

Tissue	Weight			Combustion time			Carbon		
	A	B	C	A	B	C	A	B	C
	gm	gm	gm	min	min	min	per cent	per cent	per cent
1 Blood	0.790	0.935	0.640	25	30	15	11.5	11.6	10.1
2 Spleen	0.230	0.130	0.090	10	10	5	10.8	13.3	13.9
3 Adrenals	0.010	0.010	0.010	5	5	5	13.6	18.1	33.3
4 Kidneys	0.385	0.310	0.350	10	10	10	11.9	9.6	18.4
5 Liver	1.365	1.470	1.500	20	30	30	18.0	6.1	14.5
6 Testes	0.150	0.160	0.140	10	10	10	9.9	8.7	10.4
7 Thymus	0.040	0.020	0.020	8	7	6	11.9	23.2	30.6
8 Heart	0.100	0.100	0.090	10	7	8	14.0	14.8	18.3
9 Lungs	0.135	0.140	0.140	10	7	10	13.2	13.5	13.7
10 Lymph nodes	0.040	0.020	0.060	10	7	6	16.2	23.6	16.7
11 Brain	0.390	0.430	0.430	11	15	10	10.8	8.4	13.0
12 Muscle	0.070	0.240	0.530	10	6	11	16.9	12.3	12.0
13 Stomach and small intestine	1.855*	1.900*	1.430*	25	30	30	10.9	4.3	11.2
14 Large intestine	1.440*	1.650*	2.750*	20	30	40	11.0	7.4	10.2
15 Skin and hair	2.99†	3.14‡	3.24§						
	1.27	1.72	0.45		30	20		22.0	29.2
	0.50	1.42			28			30.4	
	0.46			16			24.8		
	0.76			18			26.2		
16 Carcass	9.72	8.20¶						10.8	
	1.31	2.76		18	60		13.9		
	8.41	1.80			45				
		1.80			45				
		1.84			45				
17 Bone			0.200			10			10.0
18 Urine (bladder)		0.030			3			7.7	
" (collected)	1	0.440	1.400		10			3.0	2.6
" " "	2		0.670						3.4

\* Covered with 85 per cent phosphoric acid before combustion fluid was added

† Divided into four portions for oxidation. The ebullition of carbon dioxide was beyond control in the oxidation of the first two portions. The third and fourth parts were covered with 85 per cent phosphoric acid before combustion fluid was added, oxidation proceeded smoothly then.

‡ Divided into two parts for oxidation, each was covered with 85 per cent phosphoric acid before combustion mixture was added.

§ Only a small portion oxidized, covered with 85 per cent phosphoric acid before oxidation.

|| Oxidized in two portions. Samples covered with 85 per cent phosphoric acid before combustion mixture was added. Reaction violent in the case of the larger sample because of its extreme size, absorption of carbon dioxide incomplete.

¶ Oxidized and absorbed in four portions, combined again for precipitation of barium carbonate. Samples placed in 85 per cent phosphoric acid before oxidation.

tube The reservoirs, *H* and *I*, are of 500 and 1000 ml capacity, one is usually used to store a blank sample for background measurements, and the other is used to store discarded samples, so that many such samples can be disposed of simultaneously Although diagrammed otherwise, the leveling bulb, *G*, is permanently sealed just under the mercury reservoir, and the level of the mercury is controlled by vacuum, as is customary with McLeod gages

The counter, *A*, is of conventional design, having a copper cathode approximately 20 cm in diameter and 20 cm long and a central wire of 0.004 inch tungsten It is mounted vertically, with about 2 inches of lead shielding on top and about 1½ inches on the sides The shielding on the sides extends about 5 inches below the cathode, but there is no shielding directly

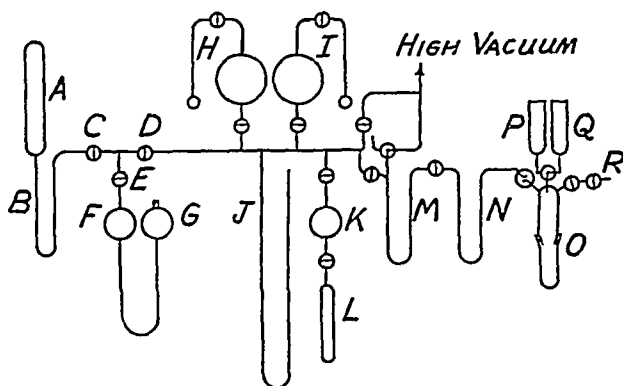


FIG 3 Apparatus used in the determination of radioactive carbon *A*, Geiger-Müller tube, *B*, trap, *F*, mixing bulb, *G*, leveling bulb, *H* and *I*, carbon dioxide reservoirs, *J*, manometer, *K*, "doser" bulb, *L*, carbon disulfide reservoir, *M* and *N*, traps, *O*, generator, *P*, perchloric acid reservoir, *Q*, water reservoir

below the tube Under these conditions, the background is about 0.75 count per second

Because the voltages required for counting with carbon dioxide are higher than are available in commercial Geiger-Müller counter circuits, a 4000 volt supply has been prepared, according to the circuit of Fig 4 Exceptional stabilization was not attempted Instead, a constant voltage transformer is used ahead of the supply, the stabilization is adequate to control the small fluctuations which pass through the transformer In the circuit, transformer  $Tr_1$  and potentiometer  $R_1$  are ganged The maximum rotation possible with the potentiometer is such that only 115 volts can be applied to the primary of high voltage transformer  $Tr_2$ , when the minimum is about 10 volts Under these conditions, the voltage applied to the regulator tube  $T_2$  is about 600 volts when the output voltage is 3500, and

is less at lower output voltages. With the voltage available from this supply, it is possible to count samples at pressures up to 35 cm. of carbon dioxide.

Since most of the samples were expected to have very low activities, of the order of background, classical resistor-condenser quenching was considered satisfactory. The quenching resistor is 500 megohms, with a 5 micro-microfarad condenser in parallel and a 20 megohm resistor in series to supply the signal to the preamplifier. A commercial scale of 32 circuit is employed.

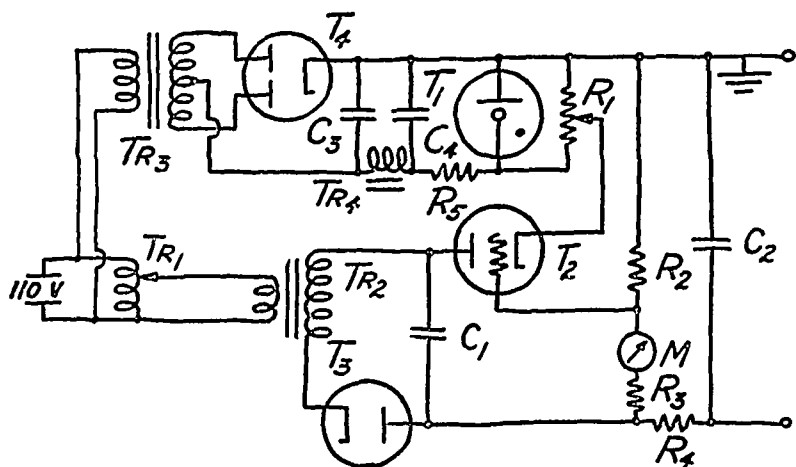


FIG. 4 Schematic diagram of high voltage supply.  $T_1$ , OC3/VR105,  $T_2$ , 6SF5,  $T_3$ , 2X2/879,  $T_4$ , 5Y3,  $Tr_1$ , Variac, type 200B connected for 0 to 135 volt secondary,  $Tr_2$ , high voltage transformer rated at 6000 volts, 2 milliamperes,  $Tr_3$ , Stancor P6289,  $Tr_4$ , Stancor C1420,  $C_1$  and  $C_2$ , 0.1 microfarad, 7000 volts,  $C_3$  and  $C_4$ , 8 microfarads, 450 volts,  $R_1$ , 20,000 ohms, general radio type 301,  $R_2$ , 120,000 ohms, 1 watt,  $R_3$ ,  $8 \times 470,000$  ohms, 1 watt,  $R_4$ , 5 megohms, 1 watt,  $R_5$ , 5000 ohms, 10 watts,  $M$ , 0 to 1 milliamperes. (In addition, the input to  $C_1$  is through a 200,000 ohms, 1 watt, resistor.)

As is to be expected with resistor-condenser quenching, the net counting rates on the plateaus of different samples do not increase linearly with the specific activities. A calibration curve is used to correct for these losses due to the dead time of the counter. The system is missing about 10 per cent of the counts when the measured counting rate is 20 counts per second, a loss rate of the correct order of magnitude for the time constant of the quenching circuit.

We have observed a second source of lost counts at counting rates above 10 to 15 counts per second. Apparently due to some sort of overdriving of the scaling and recording circuits, there results a distortion of the plateau, which, at lower rates, is usually several hundred volts in width. The

counting rate rises rapidly to a peak at a voltage a little above threshold (about 60 volts above) and then drops off with increasing voltage. The greater the true counting rate, the more rapidly the rate falls off with increasing voltage above the peak. In a typical case, at about 26 counts per second the counting rate drops over 10 per cent in the 120 volts just above the voltage corresponding to the peak.

Use of the low voltage peak counting rate obviates losses due to the overdriving. The negative slope of the plateau can be eliminated by decreasing the input resistance to the preamplifier as the overvoltage is increased, but this is not as convenient as to locate the peak. In our procedure, an operator counts at 120 volts above the threshold for 5 minutes. He then counts at 240 volts above the threshold for as much longer as is necessary to give the required precision, a time which he determines with the help of a family of curves giving the total number of counts required for several specified degrees of precision as functions of the ratio of total to background counts. If the counting rates at the two voltages agree within statistical expectations, he accepts the indicated rate. If they disagree, he counts further. From the additional counting, he may learn that the plateau is as flat as normal, in which case he accepts the corresponding counting rate, or that the plateau has a negative slope, in which case he locates the peak and uses its counting rate for calculations.

The detailed procedure for generation of  $\text{CO}_2$  (Fig. 3) is as follows. The sample of barium carbonate having been introduced into generator *O* and trap *N* having been cooled to  $-80^\circ$ , the generating system is evacuated roughly. Trap *M* is cooled to  $-195^\circ$  and the evacuation is continued until the water in the generator begins to boil. Perchloric acid, which is used because its barium salt is soluble, is then introduced slowly from reservoir *P*. When the liberation is complete, the system is again evacuated until the water boils. Three 1 ml. doses of air are admitted through *R*, and the evacuation is repeated, followed by a second dosing with air and another evacuation. The stop-cock between traps *M* and *N* is closed and the condensed carbon dioxide is exposed to the high vacuum for 3 minutes. The carbon dioxide is then allowed to evaporate into the system, measured volumetrically, and transferred to the counter for measurement of radioactivity.

Usually the dose of carbon disulfide is condensed into trap *B* during the generation of carbon dioxide. The quantity of carbon dioxide is measured in the manifold, which has a volume of about 80 ml. When both carbon dioxide and carbon disulfide have been allowed to evaporate from trap *B* into the counter tube, the mixture is not uniform, the more volatile oxide having evaporated faster than the sulfide. It has been found that five mixings by expansion into reservoir *F* produce a satisfactory mixture for counting.

Because of the long time constant of the quenching circuit, it is necessary to dilute very strongly samples of higher activity, such as those from respiration and urine in the case of radioactive urethan. As much as 100-fold dilution is sometimes required. In such a case, the use of a conveniently weighable quantity of radioactive sample necessitates using perhaps 1 liter of diluent carbon dioxide at atmospheric pressure. In order to insure the homogeneity of such a sample, the gas is tediously twice evaporated into one of the reservoir flasks and recondensed before a portion is removed for counting.

The anticipated variation between experimental animals minimized the precision required of the apparatus, and exhaustive tests of the precision have not been made. The following indications have been obtained: (1) The volume of gas, presumably carbon dioxide, recovered from barium carbonate samples and from c.p. calcium carbonate is usually within 2 per cent of that calculated on the assumption of complete purity. It should be noted, however, that complete recovery is not particularly important in this procedure, since specific activity is the property determined. For diluted samples, it is necessary only that recovery be the same for both active sample and inactive diluent. It has been our practice to generate the carbon dioxide from sample and diluent simultaneously, and to calculate the dilution factor from their weights. (2) In a series of 50 measurements of specific activity by three operators, using different quantities of two samples of barium carbonate, the results were self-consistent within a probable error of 1.9 per cent. (3) In quadruplicate determinations on each of three samples involving extreme dilution, the probable error was 1.2 per cent.

In the determinations quoted above, the probable error associated with the number of counts involved was rarely much less than 1 per cent.

#### DISCUSSION

Although one must be prepared to expect vast differences in rates of excretion, routes of excretion, and accumulation of active molecules or ions in particular tissues, depending on the compound in question and its metabolic processes, we have found that starting with activities of 2.5 microcuries per mouse contained in active urethan we have been able to determine with a fair degree of accuracy the amount of  $C^{14}$  in any 50 mg. of tissue 24 hours after injection. This has been possible with a compound which breaks down fairly rapidly (at least 85 to 95 per cent in 24 hours) and when the  $C^{14}$  is fairly evenly distributed throughout the normal animal.

Using the techniques described, we have been able to obtain reproducible tracer data on radioactive urethan with the active carbon in the carbonyl group. Results of the urethan experiments are being published elsewhere.

No attempts will be made at this time to compare the accuracy or rapidity of the assay for  $C^{14}$  in the gaseous state as opposed to measurement in the form of  $BaCO_3$ , nor to discuss the relative advantages of the Geiger counter versus the electroscope

Our principal reasons for adoption of the present procedure were (1) a desire to keep animal radiation exposure to a minimum, in which case the added sensitivity of the gaseous measurement is an advantage, and (2) the fact that without previous experience in the field and without pronounced preferences as to method we were influenced by immediate availability of necessary equipment. During the past 6 months of nearly continuous use of the described techniques, we have found them satisfactory. It has been possible to account for all of the radioactive carbon injected with an over-all accuracy of about  $\pm 10$  per cent.

#### SUMMARY

1 An apparatus designed to absorb mouse respiratory carbon dioxide suspected of containing radioactive  $C^{14}$  is described.

2 A procedure is outlined for oxidizing tissues prior to radioactive carbon assay.

3 A method is given for determination of radioactive carbon in the gaseous state.

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# SYNTHESIS AND X-RAY INVESTIGATION OF METHYL-SUBSTITUTED LONG CHAIN HYDROCARBONS RELATED TO PHTHIOCERANE

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Phthiocerol, the characteristic wax alcohol of the human and bovine types of tubercle bacillus (1-4), contains one methoxy and two hydroxy groups and has the empirical formula  $C_{35}H_{72}O_3$  or  $C_{35}H_{74}O_3$ . The alcohol is optically active,  $[\alpha]_D$  in chloroform  $-4.8^\circ$ , and melts at  $73-74^\circ$  (1, 5). From a monolayer study (6) the present authors concluded that phthiocerol was a very long molecule with one or more of the polar groups near one end. If side chains other than the methoxyl group are present, the results suggested that these are short, probably methyl groups. This view has furthermore been strengthened by the fact that the long x-ray crystal spacing of phthiocerol is  $46.2 \text{ \AA}$ .<sup>1</sup> By reduction of the iodo compound obtained in the methoxyl determination Stodola and Anderson (1) prepared the parent hydrocarbon, for which Ginger and Anderson (5) have proposed the name phthiocerane. The substance obtained by Stodola and Anderson melted at  $58.5-59.5^\circ$  and was optically inactive. An x-ray investigation by one of the present authors (7) showed, however, that this preparation was impure. The long spacing calculated from the single order observed was  $44 \pm 1.5 \text{ \AA}$  and the side spacings were, within the experimental error, the same as those of the normal form of normal chain hydrocarbons. This led to the suggestion that a straight hydrocarbon chain might be involved. Ginger and Anderson (5) subsequently purified the hydrocarbon. The phthiocerane thus obtained melted at  $59-60^\circ$ . The low melting point of the pure hydrocarbon and its high solubility in ether showed that it could not be a straight chain hydrocarbon.

As described in the experimental part, we have carried out an x-ray study on a sample of phthiocerane kindly given by Professor Anderson. Crystallized from acetone, the purified hydrocarbon gave a much better diffraction pattern than the earlier specimen, showing three clear orders of a long spacing of  $46.3 \text{ \AA}$  and strong side spacings of  $4.11$  and  $3.70 \text{ \AA}$ . As the long spacing of the normal chain  $C_{35}$  hydrocarbon (*n*-pentatriacontane) is  $46.64 \text{ \AA}$  (vertical or A form (8)), it appeared unlikely that phthiocerane with the empirical formula  $C_{34}H_{70}$  or  $C_{35}H_{72}$  could have more than

<sup>1</sup> Stenhagen, E., unpublished work.



one methyl side chain The scanty information on branched chain hydrocarbons of high molecular weight available in the literature (*cf* Egloff (9)) suggested that the position of the methyl side chain in a  $C_{34}$  or  $C_{35}$  hydrocarbon melting at  $59-60^\circ$  should be fairly close to one end of the chain By synthesizing a number of hydrocarbons with 34, 35, and 36 carbon atoms, carrying a methyl side chain in position 2, 3, etc., it thus appeared possible to settle the question of the constitution of phthiocerane

In the synthesis of long chain hydrocarbons of this type it is, of course, essential to employ methods which give the desired products free from by-products of similar molecular weight, as such by-products are either extremely difficult or impossible to remove It is furthermore very necessary that the shorter chain intermediates are free from isomeric or homologous impurities In the present case the methyl side chain has first been introduced in the appropriate position, *i e* position 2 to 5 from the hydrocarbon end of the chain, in a carboxylic acid having 17 to 19 carbon atoms The intermediate acids, the syntheses of which have been described in previous communications, were the following For the 2-methyl-substituted hydrocarbons 16-methylheptadecanoic acid (10) and 15-methylhexadecanoic acid<sup>2</sup> were used The 3-methyl-substituted compounds were synthesized starting from 15-methylheptadecanoic acid (11), while in the case of 4-methyl-substituted hydrocarbons 14-methylheptadecanoic acid (12) was employed For the 5-methyl compounds, finally, the intermediate acid was 14-methyloctadecanoic acid (12) The methyl esters (II) of the acids (I) were converted into the corresponding alcohols (III) by high pressure hydrogenation over copper-chromium oxide catalyst and the alcohols transformed into the iodides by means of iodine and phosphorus in the usual manner The iodides (IV) were used for the introduction of the corresponding alkyl radical into the appropriate normal chain  $\beta$ -keto esters (V) (13, 14) Hydrolysis and ketonic cleavage of the monoalkylation product (VI) gave long chain ketones (VII) carrying a methyl side chain in position 2 to 5 and the keto oxygen in position 18 to 20 The ketones were reduced to the corresponding hydrocarbons (VIII) by Clemmensen's method The general scheme of the syntheses is given in the accompanying flow sheet

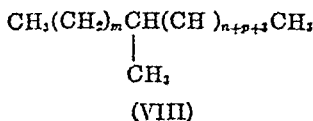
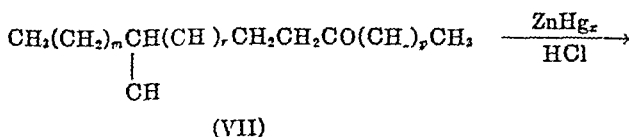
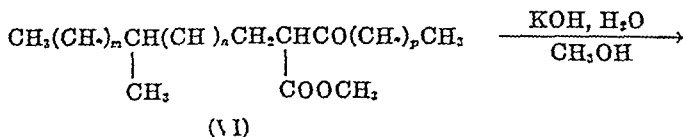
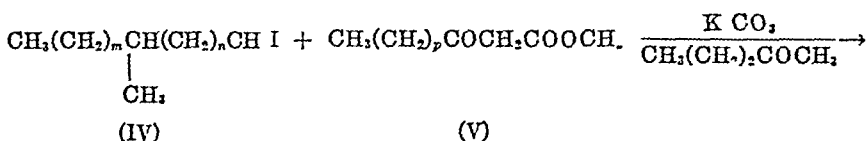
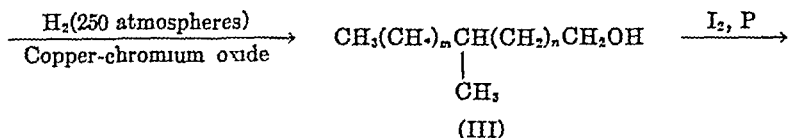
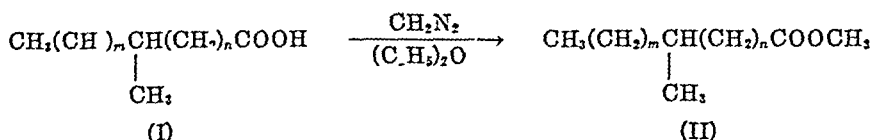
The alkylations of the  $\beta$ -keto esters (V) were carried out by means of a non-hydroxylic solvent (methyl-*n*-propyl ketone) and potassium carbonate for the formation of the potassium derivative (*cf* (13, 10)) Hydrolysis and ketonic cleavage were effected with dilute alkali at a temperature of  $50^\circ$  (12) The yield of the ketones (VII) was from 51 to 77 per cent of the theoretical, calculated on (IV) and (V), which were used in equimolecular amounts Owing to the limited amount of initial material available in

<sup>2</sup> Tägtström-Eketorp, B, unpublished work

certain cases, some ketones were prepared in very small scale (0.001 mole) runs, with satisfactory results

## EXPERIMENTAL

*Synthesis of Hydrocarbons* 15-Methylheptadecanol-1 (III,  $m = 1, n = 13$ )—Methyl 15-methylheptadecanoate (II) (25 gm, 0.084 mole), copper-



$m = 0, 1, 2, \text{ or } 3, n = 12, 13, \text{ or } 14, p = 12, 13, 14, \text{ or } 15$

chromium oxide catalyst (2 gm, cf Adkins (15)), and dioxane (13 ml) were placed in a high pressure bomb of 250 ml volume (the apparatus used was similar to that described by Adkins (16)) and the hydrogenation performed at 250°, with an initial pressure (at room temperature) of 139 kilos per sq cm. After 5 hours at 250° no further drop in pressure occurred. The bomb was allowed to cool to room temperature. The reaction mix-

ture was centrifuged, and the liquid decanted from the catalyst. The latter was washed with ether in the centrifuge and this ether added to the main portion. The solvents were distilled off and the residue (25.45 gm) distilled through a 50 cm Podbielniak column. After a small forerun (0.3 gm) distilling up to  $152^{\circ}$ , 0.8 mm, 20.85 gm of 15-methylheptadecanol-1, b.p.  $149.0\text{--}148.1^{\circ}$ , 0.6 mm, were obtained. Yield 92 per cent of the theoretical. The distilled material melted at  $26.5^{\circ}$ . The alcohol is very soluble in the common organic solvents.

<i>Analysis</i> — $\text{C}_{18}\text{H}_{38}\text{O}$ (270.5)	Calculated	C 79.94, H 14.16
	Found	" 80.43, " 14.17

*1-Iodo-15-methylheptadecane* (IV,  $m = 1$ ,  $n = 13$ )—15-Methylheptadecanol-1 (16 gm, 0.0592 mole), red phosphorus (0.6 gm, 0.019 atom), and iodine (8.0 gm, 0.063 atom) were placed in a 100 ml round bottomed flask fitted with reflux condenser and calcium chloride tube and the mixture heated in an oil bath to a temperature of  $145\text{--}150^{\circ}$  for 3 hours, with occasional shaking. After cooling to room temperature 50 ml of ether were added and the mixture agitated. The ether layer was decanted and the bottom layer extracted twice with small portions of ether. The combined ether solutions were freed from phosphorus by filtration and then washed with water and dried with sodium sulfate. During the evaporation of the ether about one-third of the solution was lost by accident. The residue (13.5 gm) was distilled, giving 0.3 gm of a forerun distilling up to  $175^{\circ}$ , 1.0 mm, and 12.6 gm of colorless 1-iodo-15-methylheptadecane, b.p.  $175\text{--}178^{\circ}$ , 1.0 to 1.1 mm. Yield (not corrected for loss) 56 per cent of the theoretical.

<i>Analysis</i> — $\text{C}_{18}\text{H}_{37}\text{I}$ (380.4)	Calculated, I 33.34, found, I 32.88
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*14-Methylheptadecanol-1* (III,  $m = 2$ ,  $n = 12$ )—Methyl 14-methylheptadecanoate (12) (11.2 gm, 0.0378 mole) was reduced in the manner just described for the 15-methyl-substituted isomer. Distillation through a 50 cm Podbielniak column gave, after a forerun (0.4 gm) distilling up to  $158^{\circ}$ , 1.2 mm, 8.5 gm of 14-methylheptadecanol-1, b.p.  $158.0\text{--}158.2^{\circ}$ , 1.2 mm, in the form of a colorless liquid. Yield 83 per cent of the theoretical.

<i>Analysis</i> — $\text{C}_{18}\text{H}_{38}\text{O}$ (270.5)	Calculated	C 79.94, H 14.16
	Found	" 79.96, " 14.16

*1-Iodo-14-methylheptadecane* (IV,  $m = 2$ ,  $n = 12$ )—14-Methylheptadecanol-1 (6.85 gm, 0.0263 mole) was converted into the iodo derivative in the manner described for the 15-methyl-substituted isomer, by the use of 0.27 gm of red phosphorus and 3.55 gm of iodine. Distillation under

reduced pressure gave, after a forerun distilling up to  $146^{\circ}$ , 3 mm, 8.65 gm of a slightly yellow-colored 1-iodo-14-methylheptadecane, b p  $146-149^{\circ}$ , 0.3 mm. Yield 89 per cent of the theoretical.

*Analysis*— $C_{15}H_{31}I$  (380.4) Calculated, I 33.34, found, I 33.30

*14-Methyloctadecanol-1* (III,  $m = 3$ ,  $n = 12$ )—Methyl 14-methyloctadecanoate (12) (6.7 gm, 0.0216 mole) was reduced in the manner described above. The residue obtained after working up the reaction mixture gave on distillation a forerun (0.2 gm) distilling up to  $156^{\circ}$ , 0.7 mm, followed by 4.2 gm of 14-methyloctadecanol-1, b p  $156.0-156.3^{\circ}$ , 0.7 mm. Yield 68 per cent of the theoretical.

*Analysis*— $C_{17}H_{34}O$  (284.5) Calculated C 80.24, H 14.18  
Found " 80.29, " 14.22

*1-Iodo-14-methyloctadecane* (IV,  $m = 3$ ,  $n = 12$ )—3.75 gm of 14-methyloctadecanol-1 were converted into the iodo derivative, by the use of 0.15 gm of red phosphorus and 1.84 gm of iodine. The reaction mixture was worked up and the residue distilled. After 0.25 gm of forerun, b p up to  $148^{\circ}$ , 0.2 mm, 4.3 gm (82 per cent of the theoretical) of 1-iodo-14-methyloctadecane, b p  $148-151^{\circ}$ , were obtained as a nearly colorless liquid.

*Analysis*— $C_{15}H_{31}I$  (394.4) Calculated, I 32.15, found, I 31.99

*Synthesis of Ketones*—The procedure used was in all cases the same, with unimportant variations only, and we therefore limit the description of the syntheses to one typical example. The figures for the yields of the various ketones are included in Table IV, and the analyses are given in Table I.

*4-Methyltrtriacontanone-19* (VII,  $m = 2$ ,  $n = 12$ ,  $p = 13$ )—1-Iodo-14-methylheptadecane (1.0 gm, 0.00263 mole), methyl 3-ketoheptadecanoate (14) (0.8 gm), methyl-*n*-propyl ketone (5 ml, the commercial product (Shell) was dried by means of potassium carbonate and distilled immediately before use), and potassium carbonate (1.5 gm, analytical grade, dried by ignition in an open porcelain dish over a Bunsen burner) were placed in a 25 ml round bottomed flask provided with a reflux condenser and calcium chloride tube (before assembling, all glass parts were thoroughly dried at  $110^{\circ}$ ) and the mixture boiled under a reflux on a sand bath for 18 hours. Considerable bumping sometimes takes place and the glass parts of the apparatus should therefore be tied together in some way. After cooling, the mixture was poured into excess 5 per cent sulfuric acid, ether added, and the organic layer washed twice with water. The organic layer was dried by means of sodium sulfate and the solvents removed under reduced pressure. The slightly yellow alkylation product was hydrolyzed and subjected to ketonic cleavage by adding a solution of potassium hydrox-

ide (4 gm) in water (4 ml) and methanol (60 ml) and keeping the mixture at a temperature of 50° for 20 hours, with occasional shaking. The mixture was then poured into excess 5 per cent sulfuric acid and the resulting mixture extracted with ether. The ether solution was washed with water and dried (sodium sulfate). The ether was removed and the residue, a yellow solid, crystallized from acetone. The yield of practically white, microcrystalline material was 1.30 gm or 67 per cent of the theoretical. One recrystallization from acetone of the material thus obtained raised the melting point by 0.7°, but further recrystallization caused no change.

The data for the ketones given in Table IV are for material crystallized two or three times from acetone. The ketones are sparingly soluble in

TABLE I  
*Analysis of Ketones*

Compound	Formula	Per cent composition			
		Found		Calculated	
		C	H	C	H
2-Methyltriotriacontanone-18	$C_{34}H_{68}O$	82.74	13.86	82.85	13.91
3-Methyltriotriacontanone-19		82.84	13.98		
4-Methyltriotriacontanone-19		82.92	13.88		
5-Methyltriotriacontanone-20		82.99	13.91		
2-Methyltetraotriacontanone-19	$C_{35}H_{70}O$	82.65	13.82	82.92	13.92
3-Methyltetraotriacontanone-19		82.94	13.83		
4-Methyltetraotriacontanone-19		83.11	13.91		
5-Methyltetraotriacontanone-20		82.93	13.93		
2-Methylpentatriacontanone-18	$C_{36}H_{72}O$	82.88	13.86	83.00	13.93
3-Methylpentatriacontanone-19		82.94	13.83		
4-Methylpentatriacontanone-19		83.15	13.91		
5-Methylpentatriacontanone-20		83.15	13.85		

cold, but dissolve in boiling acetone and ethanol. They are fairly soluble at room temperature in benzene and chloroform.

*Reduction of Ketones*—The ketones were reduced to the corresponding hydrocarbons by Clemmensen's method in the manner described below.

The ketone (1 gm), amalgamated granulated zinc (60 gm), concentrated hydrochloric acid (50 ml), and glacial acetic acid (10 ml) were boiled under a reflux. Three times daily the mixture was cooled, the liquid decanted, and fresh zinc (10 gm), hydrochloric acid (50 ml), and glacial acetic acid (10 ml) added. The total boiling time was 80 to 90 hours.

*Purification of Hydrocarbons*—The crude hydrocarbons from the reduction may contain unsaturated material formed during the reduction and some unchanged ketone. Most of the latter may be removed by crys-

tallization from acetone, in which the hydrocarbons are considerably less soluble than the ketones. Further purification was carried out by treating the hydrocarbons with concentrated sulfuric acid at a temperature of 125–130° for 1 to 2 hours, as described by Piper *et al.* (8). During the first treatment in this way considerable darkening occurred and the melting point of the recovered hydrocarbon was, after crystallization from acetone, 0.5–1.0° higher than before the treatment. The process had to be repeated two to three times before the sulfuric acid in contact with the sample showed no darkening, but these later treatments in most cases did not cause any further change in the melting point of the hydrocarbon.

Finally, some of the hydrocarbons (see Table VI) were subjected to molecular distillation in a still of the type made by Schott and Genossen (17). In order to distil small quantities of hydrocarbons in this apparatus, a special inset of aluminum carrying a small flat platinum cup to hold the distilland had to be used together with the vessel intended for sublimation experiments. It was thought that molecular distillation might remove any bimolecular reduction products. In no case did molecular distillation followed by recrystallization change the melting point or other properties of a hydrocarbon to a perceptible extent, however. The analyses of the hydrocarbons are given in Table II.

The hydrocarbons are less soluble in boiling acetone than the corresponding ketones, but are fairly soluble in ethyl ether at room temperature. When crystallized from solvents such as acetone or ethyl acetate, the 2-methyl compounds form small plates which on the filter are packed together to flakes with a definite luster. The 3-, 4-, or 5-methyl-substituted hydrocarbons crystallize in the form of very small crystals. On the filter the soft crystal mass is packed together to a white mass which in the case of the 3-methyl compounds shows a definite luster, but in the 4- and 5-methyl hydrocarbons shows a weak luster only and no macroscopic crystallinity.

### *X-ray and Thermal Investigations*

*Technique*—The X-ray investigation was carried out with nickel-filtered Cu  $K_{\alpha}$  radiation<sup>3</sup>. For work at room temperature (20–22°) a simple goniometer with flat photographic films was employed. Powder methods had to be used, as it does not appear possible to obtain well formed crystals of sufficient size for single crystal work from the material under examination. The ketones or hydrocarbons were spread out in a thin layer on small glass plates (6 × 20 mm, cut from microscope object glasses). The layers were prepared by pressing out the crystalline material, by melting the substance onto the glass plate by means of the hot wire technique.

<sup>3</sup>  $\lambda = 1.5418 \text{ \AA}$ , cf. notice (*J. Am. Chem. Soc.*, 69, 2919 (1947)).

(18), or by depositing wet crystals on the plate. In the last case the compound was dissolved in hot acetone and the solution allowed to cool slowly to room temperature. The crystalline material that separated out was suspended in the mother liquid and the resulting pulp dropped onto the glass plate. On evaporation of the solvent thin even layers resulted. The diffraction patterns obtained from these layers showed very sharp and distinct orders of the long spacing but the side spacings were usually very weak or absent. In order to bring out the latter, specimens prepared by one of the other methods had to be used. In some cases the side spacings were obtained from diffraction patterns given by specimens in very thin walled glass capillary tubes (Keesom capillaries<sup>4</sup> (19)). The glass plates

TABLE II  
*Analysis of Hydrocarbons*

Compound	Formula	Per cent composition			
		Found		Calculated	
		C	H	C	H
2-Methyltrtriacontane	$C_{34}H_{70}$	85.15	14.70	85.27	14.73
3-Methyltrtriacontane		85.44	14.56		
4-Methyltrtriacontane		85.41	14.66		
5-Methyltrtriacontane		85.29	14.63		
2-Methyltetraatriacontane		85.36	14.75		
3-Methyltetraatriacontane	$C_{36}H_{72}$	85.21	14.73	85.28	14.72
4-Methyltetraatriacontane		85.18	14.70		
5-Methyltetraatriacontane		85.31	14.74		
2-Methylpentatriacontane		85.31	14.67		
3-Methylpentatriacontane		85.00	14.65		
4-Methylpentatriacontane	$C_{36}H_{74}$	85.03	14.65	85.29	14.71
5-Methylpentatriacontane		85.07	14.66		

were mounted with the surface carrying the specimen along the axis of the spectrograph and rocked over an angle of  $7^\circ$  during exposure. The distance from specimen to photographic film was 10 or 15 cm. Ilford X-ray ("red seal") film and Agfa "Laue" film were used, the spacings given in Tables III, V, and VII have been corrected for film shrinkage.

Diffraction patterns at temperatures above room temperature were obtained with another goniometer, which carried a thermostat similar to that used by Muller (20). The thermostat was made from a solid copper rod 5 cm in diameter. The openings for the passage of the X-ray beam were covered by thin cellophane. Transformer oil from a well stirred bath, that could be set to any desired temperature by means of heaters

<sup>4</sup> Obtained from Messrs. Hanff and Buest, Berlin, Germany.

regulated by a contact thermometer and relay, was pumped through the hollow outer mantle of the goniometer thermostat via flexible, oil-resistant tubing. In the upper surface of the copper thermostat there was a threaded hole about 2 cm in diameter into which a threaded copper rod carrying the specimen plate could be screwed. The temperature of the specimen was read on an Anschütz type thermometer placed in a deep hole in the central copper rod. The specimen could be held at the desired temperature within 0.1–0.2° for indefinite periods, provided that the room temperature was reasonably constant. The copper thermostat could be rocked through angles of 5°, 10°, or 20° during exposure. In order to follow changes in the diffraction patterns with temperature, provision was made for recording up to ten different diffraction patterns on the same film by using a shield with a slit with a height of 6 mm in front of the film and displacing the latter a distance equal to the height of the slit on changing to another temperature. The film distance was 5 or 10 cm and the minimum time of exposure needed was about 10 minutes, with a Philips-Muller tube running at 15 to 20 milliamperes.

The melting and solidification of the hydrocarbons and ketones was studied with specimens in thin walled capillary tubes of about 1 mm internal diameter. The tubes were sealed off and attached to an Anschütz type thermometer (graduated in 0.2° and having a range of 50–100°) in such a way that the specimen was in a position close to the mercury bulb, and the whole immersed in a well stirred Nujol bath contained in a glass beaker. The thermometer used was compared carefully with the laboratory standard. If the heating and cooling of the bath were carried out very slowly, it was possible to reproduce melting and solidification points within 0.1° or better. The specimens were observed through a binocular microscope with stereoscopic vision, having a magnification of 15 ×.

### Results

*Ketones*—The x-ray and thermal data are collected in Tables III and IV. The C<sub>34</sub> and C<sub>35</sub> 2-methyl-substituted ketones have two different crystal modifications which differ considerably in melting point. The x-ray study, which in the case of ketones has been limited to an examination of pressed and melted specimens at room temperature, shows that the higher melting modification has vertical molecules and the lower melting form tilted molecules. For the C<sub>36</sub> compound only one crystalline form with tilted molecules has been observed. The behavior of the 3-, 4-, and 5-methyl-substituted ketones varies apparently in a somewhat irregular manner with the position of the side chain and the keto group, both vertical and tilted forms being observed. Two of the 4-methyl-substituted ketones appear to be able to exist in three different crystalline modifications.



TABLE III

*Crystal Spacings for Methyl-Substituted Ketones*

The x-ray spacings are in Angstrom units

Compound	Crystallized from acetone		Melted	
	Long spacing	Side spacings	Long spacing	Side spacings
2-Methyltritracontanone-18	45 3 V *	4 15, 3 75	45 3 V and 35 8	2 sets of spacings
2-Methyltetracontanone-19	47 2 "	4 10, 3 74	47 2 V	4 10, 3 74
2-Methylpentatriacontanone-18	34 5	3 83, 3 50	34 5	3 83, 3 50
3-Methyltritracontanone-19	45 9 V	4 11, 3 74	45 9 V	4 11, 3 74
3-Methyltetracontanone-19	47 2 "	4 14, 3 76	47 2 "	4 14, 3 76
3-Methylpentatriacontanone-19	48 8 "	4 12, 3 75	36 5	4 59, 3 76
4-Methyltritracontanone-19	45 5 "	4 11, 3 75	45 5 V	4 14, 3 75
4-Methyltetracontanone-19	41 9	4 15, 3 74	47 2 " and 39	2 sets of spacings
4-Methylpentatriacontanone-19	39 5	4 14, 3 72	42 7 and 35 8	" "
5-Methyltritracontanone-20	45 7 V	4 09, 3 74	46 3 V	4 12, 3 75
5-Methyltetracontanone-20	47 3 "	4 12, 3 75	48 V	4 13, 3 75
5-Methylpentatriacontanone-20	43 5	4 14, 3 75	48 9 V	4 12, 3 75

\* V indicates vertical form

TABLE IV

*Yield and Thermal Data for Ketones*

Compound	Yield	M p		Solidification point
		Crystallized from acetone	After previous melting	
	per cent	°C	°C	°C
2-Methyltritracontanone 18	51	75 8-76 0	70 5	70 3
2-Methyltetracontanone-19	62	78 8-79 2	72 8	72 5
2-Methylpentatriacontanone-18	53	74 4-74 6	74 4-74 6	74 1
3-Methyltritracontanone-19	68	72 5	70 5	69 3
3-Methyltetracontanone-19	51	69 9-70 1	68 3	68 2
3-Methylpentatriacontanone-19	59	69 3-69 5	68 9	68 8
4-Methyltritracontanone-19	67	65 2-65 4	63 9	63 8
4-Methyltetracontanone-19	72	66 0	64 2	64 2
4-Methylpentatriacontanone 19	73	66 2-66 4	65 2	65 1
5-Methyltritracontanone-20	77	63 6-63 7	62 7	62 6
5-Methyltetracontanone-20	67	63 3	63 3	63 2
5-Methylpentatriacontanone-20	59	64 0-64 2	64 0	63 9

The ketones gave in general good diffraction patterns showing at least seven to nine 00l reflections, with alternation in the intensities (even orders weak) owing to the presence of the keto group near the middle of the chain

The appearance of crystalline modifications in which the chains are tilted is in marked contrast to the behavior of normal chain ketones which have only been found to crystallize with vertical chains (21)

*Hydrocarbons*—The results of the x-ray investigation of the synthetic methyl-substituted hydrocarbons and of phthiocerane are summarized in Table V and the thermal data in Table VI

TABLE V  
*X-ray Data for Methyl-Substituted Hydrocarbons*

The x-ray spacings are in Angstrom units

Compound	Low temperature form*			High temperature form†		
	Long spacing	Side spacings		Long spacing	Side spacings	
2-Methyltrtriacontane	35.6	4.12, 3.70, 3.94		38.7	4.40, 4.08, 3.89	
2-Methyltetraatriacontane	33.8	4.12, 3.66		39.6	4.41, 4.07, 3.88	
2-Methylpentatriacontane	37.6	4.13, 3.71, 3.95		41.1	4.41, 4.08, 3.90	
				40.3‡	4.23, 4.08, 3.95, 3.76	
3-Methyltrtriacontane	46.0	4.12, 3.71		40.5	4.39, 3.96, 4.09	
3-Methyltetraatriacontane	47.1	4.12, 3.70		40.0	4.39, 3.95, 4.11	
3-Methylpentatriacontane	48.6	4.12, 3.71		42.2	4.37, 3.93, 4.11	
4-Methyltrtriacontane	46.3	4.11, 3.70		42.2	4.36, 3.97§	
4-Methyltetraatriacontane	47.7	4.12, 3.70		42.5	4.36, 3.98§	
4-Methylpentatriacontane	49.2	4.12, 3.70		44.0	4.36, 4.00§	
5-Methyltrtriacontane	47.0	4.12, 3.70		42.2	4.36, 4.00§	
5-Methyltetraatriacontane	48.2	4.11, 3.69		42.8	4.35, 4.00§	
5-Methylpentatriacontane	49.6	4.11, 3.70		44.0	4.35, 4.01§	
Phthiocerane	46.3	4.11, 3.70		42.4	4.36, 4.03§	
Equimolecular mixture of 3-methyl- and 5-methyl- trtriacontane	46.4	4.11, 3.70		41.6	4.37, 4.03§	
Equimolecular mixture of 4-methyltrtri- and 4- methylpentatriacontane	47.3	4.11, 3.70		42.8	4.35, 4.02§	

\* At room temperature (20–22°)

† Near the transition point

‡ Intermediate form at a temperature just below the transition to the high temperature form

§ Diffuse reflection

The 2-methyl-substituted compounds crystallize from acetone in tilted forms which give very good x-ray diffraction patterns showing up to ten orders of the long spacing. With increase in order there is a regular decrease in the intensity of the reflections. The values for the long spacings of the low temperature form given in Table V, which are probably correct to 0.25 Å, indicate that there is alternation between members having an

odd and an even number of carbon atoms. When the temperature is raised above room temperature, there is a slight increase in the side spacings, with practically no change in the long spacing until a transition to a high temperature form with a smaller tilt takes place. For 2-methyltrtriacontane and 2-methylpentatriacontane this transition occurs at about 11° below the melting point, while in the case of 2-methyltetratriacontane

TABLE VI  
*Thermal Data for Methyl-Substituted Hydrocarbons*

Compound	Transition point* on heating	M p	Solidification point
	°C	°C	°C
2-Methyltrtriacontane	55.4	66.1-66.3	65.9
2-Methyltetratriacontane	50-55†	68.1-68.3	68.0
2-Methylpentatriacontane	58.0-60.6	69.9-70.1	69.7
3-Methyltrtriacontane	48.9	61.8-61.9	61.7
3-Methyltetratriacontane‡	51.9-52.8	64.1-64.2	64.0
3-Methylpentatriacontane	55.5-56.6	66.0-66.1	65.9
4-Methyltrtriacontane‡	45.6-46.4	58.6-58.7	58.5
4-Methyltetratriacontane‡	49.4-50.6	60.7-60.9	60.6
4-Methylpentatriacontane‡	53.2-53.9	63.0-63.1	62.9
5-Methyltrtriacontane	41.5-42.8	55.5-55.7	55.5
5-Methyltetratriacontane	45.4	58.0-58.1	57.9
5-Methylpentatriacontane	48.2-49.1	60.3-60.4	60.2
Phthiocerane	45.6-46.8	59.1-59.3	59.0
Equimolecular mixture of 3- and 5-methyltrtriacontane	44.2-45.3	59.1-59.3	59.0
Equimolecular mixture of 4-methyltrtriacontane and 4-methylpentatriacontane	48.8-49.6	60.8-60.9	60.7

\* Determined from x-ray diffraction patterns taken at temperature intervals of about 1°. The temperatures between which the transition has been found to occur are given. One figure only means that both crystal forms have been observed at the temperature in question.

† Both forms were found to coexist over this temperature range.

‡ Distilled in the molecular still.

the high temperature form begins to appear at about 18° below the melting point.

As is to be expected, the high temperature form gives somewhat weaker diffraction patterns than do the low temperature forms. About seven clear orders of the long spacing are observed, the intensity of the reflections decreasing in a regular manner. The side spacings are, within the experimental error, the same for the three hydrocarbons studied and this, together with the regular increase of the long spacing and the melting point, indicates that odd and even members in this case have the same crystal

form On cooling 2-methylpentatriacontane a transition takes place on passing through the point where the transition to the high temperature form takes place on heating, but now a third crystalline modification appears The tilt of the molecules is intermediate between those of the high and low temperature forms On further cooling the intermediate form changes into the low temperature form, which at room temperature shows spacings identical with those given by material originally obtained by crystallization from acetone

The 3-, 4-, and 5-methyl-substituted hydrocarbons differ markedly in crystal behavior from the 2-substituted isomers The low temperature form obtained by crystallization from acetone has vertical molecules It is known from the work of Müller (22) (*cf* also Hengstenberg (23), Kohlhaas and Soremba (24), and Bunn (25)) that the so called A form of *n*-hydrocarbons is orthorhombic The chains are running parallel to the *c* axis, and the lengths of the *a* and *b* axes are 7.40 to 7.45 and 4.93 to 4.97 Å respectively While other crystal forms are known for the *n*-hydrocarbons (monoclinic and triclinic (22)), the normal chain ketones crystallize only in the orthorhombic form, the introduction of the keto group causing only a slight altering in the length of the short axes A comparison of the side spacings given by the vertical form of the methyl-substituted hydrocarbons and ketones shows that the crystal structure must be identical with that of the normal chain compounds, as there is a close agreement between the *h*00 reflections both as regards relative intensities and the numerical values of the spacings The data given in Table VII show that the methyl group causes a very small increase in the length of the short axes The main effect of the methyl side chain is an elongation of the *c* axis and some disorder in the direction of this axis, indicated by the rapid falling off in intensity and disappearance of the 00*l* reflections of higher order The 3-methyl compounds show four and the 4- and 5-methyl-substituted compounds three orders of the long spacing The few orders observed at small reflection angles do not allow the long spacings to be determined with a high degree of accuracy The data given in Table V are probably correct to 0.7 Å The spacings are longer than the long spacings of the normal chain isomers and there is also a slight increase in the long spacing between the 3- and the 5-methyl compounds As the side spacings are the same, this means a decrease in density which is paralleled by a slight increase in the softness of the material

Increase in temperature causes an increase in the side spacings and a decrease in the long spacings, which become more pronounced when the transition to the high temperature form is approached The temperature at which the transition occurs appears quite reproducible on slow heating, but on cooling there is a certain tendency for the high temperature form

to be metastable at lower temperatures. This tendency is strongest for the 3-methyl and least pronounced for the 5-methyl compounds. For instance, it will be found that in specimens prepared by the hot wire technique, in which the specimen is cooled rapidly, the long spacing at room temperature is in the case of 3-methyl compounds that of the high temperature form. The 4-methyl compounds, including also the equimolecular mixture of 4-methyltriacontane and 4-methylpentatriacontane, show the presence of both forms (two sets of lines in the diffraction patterns), while the 5-methyl compounds show the low temperature form only. The

TABLE VII

*Effect of Methyl Side Chain on Short Crystal Spacings of Orthorhombic Form*

Compound	Spacings calculated from $hk0$ reflections						Calculated length of axes of orthorhombic unit cell		Cross-section of unit cell
	110	200	210	020	310	220	$a$	$b$	
	$A$	$A$	$A$	$A$	$A$	$A$	$A$	$A$	$sq\ A$
4-Methyltriacontane	4 115	3 70	2 97	2 47	2 21	2 06	7 40	4 95	36 65
<i>n</i> -Hentriacontane*	4 10	3 70	2 96	2 465	2 20	2 05	7 40	4 93	36 45
4-Methyltriacontanone-19	4 115	3 75	3 00	2 465	2 225	2 06	7 50	4 93	36 9
Hentriacontanone-12†	4 11	3 73	2 96	2 45	2 21	2 055	7 46	4 90	36 6

\* Prepared by reduction of hentriacontanone-12. After purification by means of sulfuric acid in the usual manner, the hydrocarbon had within  $0.1-0.2^\circ$  the same melting point and transition points as those reported by Piper *et al.* (8). The long x-ray spacing was 41.45 Å compared with a value of 41.55 Å reported by the authors just cited.

† This ketone was synthesized in a yield of 73 per cent of the theoretical from methyl 3-ketotetradecanoate and *n*-octadecyl iodide. Hentriacontanone-12 melted at  $77.6-77.8^\circ$  and gave on analysis C 82.97, H 13.74, calculated for  $C_{31}H_{62}O$ , C 82.59, H 13.86.

mixture of 3- and 5-methyltriacontane behaves as the 4-methyl compounds do. The long spacings of the high temperature form of the  $C_{35}$  compounds are closer to those of the  $C_{31}$  than to those of the  $C_{36}$  hydrocarbons. The differences are within the experimental error, but may be due to alternation.

The introduction of a methyl side chain thus completely alters the high temperature behavior of a long chain hydrocarbon. It is well known from the work of Muller (20) that *n*-hydrocarbons show a transition point several degrees below the melting point at which the orthorhombic structure changes abruptly or gradually into a hexagonal packing of vertical chains.

which perform strong oscillation or free rotation. The methyl side chain evidently prevents the rotation of the chains, the stable high temperature crystal structure instead being one with non-rotating tilted chains.

In contrast to the 2-methyl-substituted hydrocarbons the isomers with methyl groups in positions 3, 4, and 5 show distinct annealing effects. When, for example, a specimen of 3-methylpentatriacontane crystallized from acetone is heated from room temperature, the long spacing decreases from 48.6 to 47.1 Å just below the transition point, the spacings of the (110) and (200) planes increasing at the same time by about 0.02 Å. On cooling to room temperature the long spacing increases to 47.7 Å and the side spacings return to their former values. If the specimen is heated again, but this time to a temperature just above the transition point, and allowed to cool, the long spacing at room temperature is 44.5 Å, but there is only a just perceptible increase in the side spacings compared with the specimen crystallized from acetone. The annealing thus causes a marked increase in density of the hydrocarbon. Similar results are given by the 4- and 5-methyl-substituted hydrocarbons, although the changes are somewhat less marked. Annealing effects also occur in the case of normal chain hydrocarbons (26) but do not seem to be of the order of magnitude encountered with the methyl-substituted compounds.

*Thermal Behavior of Synthetic Hydrocarbons*—When heated in melting point tubes, the hydrocarbons, which appear white and opaque at room temperature, gradually become somewhat translucent. The transition to the high temperature form appears visually as a slight clearing up in the crystal mass. It is best observed on heating and with specimens that have been melted previously, but the phenomena are much less marked than the transitions of normal chain hydrocarbons and it is very difficult to determine the exact transition temperature from visual observations. Fusion takes place over a temperature range of 0.1–0.2°.

When the 2-methyl-substituted hydrocarbons solidify, long transparent needles appear throughout the whole mass, complete solidification taking place over a temperature range of about 0.1°. On further cooling the material contracts, giving an inverted cone air gap in the center of the tube, and the material becomes translucent rather than transparent. The transition to the low temperature form on further cooling is very difficult to observe visually.

The 3-, 4-, and 5-methyl-substituted hydrocarbons solidify in a manner different from that of the 2-methyl compounds. On slow cooling of the melt there suddenly appear clusters of small crystals at several points within the melt and these clusters grow rapidly until the whole tube is filled with a white crystalline mass. The temperature range of setting is about 0.1°, and no inverted cone structure is seen on further cooling. The transition to the low temperature form is very difficult to observe.

Supercooling has not been observed for any of the hydrocarbons, the same solidification point being observed with and without a nucleus

*Comparison between Phthiocerane and Synthetic Hydrocarbons*—As already mentioned in the introduction, the purified phthiocerane, crystallized from acetone, gives at room temperature a diffraction pattern showing three orders of a long spacing of 46.3 Å and two strong side spacings of 4.11 and 3.70 Å respectively. At a temperature of 46° a transition to a tilted high temperature form takes place. The high temperature form has a long spacing of 42.4 Å and side spacings of 4.36 and 4.03 Å. The behavior of phthiocerane is thus very similar to the hydrocarbons with a methyl side chain in position 3-, 4-, or 5. Phthiocerane is a  $C_{34}$  (or possibly  $C_{30}$ ) hydrocarbon, and the melting point data given in Table VI therefore indicate that the structure is that of a 4-methyl-substituted hydrocarbon. Specimens prepared by the hot wire technique also behave as do 4-methyl compounds in showing the long spacings of both crystalline forms. The x-ray data are almost identical with those of 4-methyltrtriacontane, and the transition point is the same within the experimental error (0.5–1°). There is a small difference in the melting point, phthiocerane melting 0.5° higher than the synthetic hydrocarbon, but the general behavior on melting and solidification is exactly the same. It is very unlikely that the observed difference in melting point is due to impurity in the synthetic hydrocarbon. The x-ray diffraction patterns indicate, as a matter of fact, that the latter is somewhat better crystalline than phthiocerane. The intensity of the 001 reflections falls off more rapidly in case of phthiocerane and specimens of this hydrocarbon prepared by the crystal pulp method show strong side spacings ( $hkl0$  reflections), indicating a less orderly orientation of the crystallites with respect to the glass plate than in the case of the synthetic compound.

The methyl-substituted hydrocarbons are sufficiently alike to form solid solutions on mixing, and it is possible to prepare artificial mixtures (*cf.*, for example, the data for the equimolecular mixture of 3- and 5-methyltrtriacontane given in Tables V and VI), the thermal and x-ray data of which are quite close to those of phthiocerane. Conclusive evidence that phthiocerane is a 4-methyl-substituted hydrocarbon has been obtained, however, from a study of the infra-red absorption spectra of phthiocerane and the synthetic hydrocarbons, carried out by Dr. G. B. B. M. Sutherland and his collaborators at the Department of Colloid Science, Cambridge, England. It was found (27) that hydrocarbons with the methyl side chain in different positions could be distinguished by their infra-red absorption spectra and that the spectrum of phthiocerane was identical with that of the 4-methyl compounds.

Many of the long normal chain primary alcohols found in natural waxes

are mixtures of closely related even numbered homologues (*cf* (8)), and the wax alcohols of the timothy grass (28), the leprosy (29), and the avian tubercle bacillus (30) are the similarly related secondary alcohols (+)-octadecanol-2 and (+)-eicosanol-2. The possibility that phthiocerane is such a mixture must therefore be considered. Ginger and Anderson (5) subjected the hydrocarbon to molecular distillation and found no evidence of inhomogeneity. We have studied the behavior of a synthetic mixture of 3-methyltritriacontane and 3-methylpentatriacontane in the molecular still. The mixture used had the same solidification point as pure 3-methyltetratriacontane, *viz* 64.0°, the two components being present in very nearly equimolecular proportions. Recrystallization of this mixture from acetone did not change the solidification point. After distilling about 40 per cent of the material introduced into the still, the distillate was collected and crystallized from acetone. The solidification point of the material thus obtained was 63.6°. The residue in the still had, after similar treatment, a solidification point of 64.3°. The separation effected is sufficient to show that the possibility that phthiocerane is a mixture of 4-methyldotriacontane and 4-methyltetratriacontane in about equal proportions can be excluded. On the other hand, the detection of a very small amount of 4-methylpentatriacontane in 4-methyltritriacontane would be rather difficult. About 6 per cent of the higher homologue will raise the melting point to that of phthiocerane.

Another possible cause of the slight differences found between phthiocerane and the synthetic 4-methyltritriacontane may be that the reduction of phthiocerol has not resulted in a completely racemized hydrocarbon. A small residual optical activity in a compound of this type, which has to be examined in dilute solution, might easily escape detection.

We are greatly indebted to Professor R. J. Anderson for a specimen of phthiocerane and for his interest in the work. The microanalyses were performed by Mr. W. Kirsten in the microanalytical laboratory of this institute. The expenses involved in this work have been defrayed by grants from the National Swedish Antituberculosis Association and from the Rockefeller Foundation.

#### SUMMARY

In order to settle the question of the constitution of phthiocerane, the synthesis of a series of methyl-substituted hydrocarbons, having a total of 34, 35, or 36 carbon atoms and with the side chain in position 2, 3, 4, or 5 from the end of the chain, has been undertaken.

The crystal structure and the thermal behavior of the hydrocarbons are described. In particular, it is found that at room temperature the



2-methyl-substituted compounds exist in crystal forms in which the long chains are inclined (monoclinic or triclinic forms), while the crystal structure of the 3-, 4-, and 5-methyl-substituted hydrocarbons at this temperature is the orthorhombic structure found in the case of normal chain hydrocarbons. The behavior at high temperature of the branched chain hydrocarbons is quite different from that of the latter, however. At 10–15° below the melting point the methyl-substituted compounds show a transition to a crystal structure with tilted chains (monoclinic or triclinic form) which persists up to the melting point.

Phthiocerane behaves in the manner just described for the 3-, 4-, and 5-methyl-substituted hydrocarbons. The x-ray and thermal data as well as a comparison of the infra-red absorption spectra show that phthiocerane is a 4-methyl-substituted hydrocarbon. The x-ray data are in close agreement with those of 4-methyltriacontane, but there are some minor differences in the appearance of the diffraction patterns, and phthiocerane melts 0.5° higher than does the synthetic hydrocarbon. Some possible causes of these differences are discussed.

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# A COLORIMETRIC REACTION OF CHLORIDE ION\*

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It has been found that the reaction of halogens with certain alkaloids in the presence of persulfate may be used for the determination of chloride, bromide, and iodide ions. In the present report, the determination of chloride will be discussed.

## EXPERIMENTAL

*Specificity of Reactions*—Several alkaloids were tested for their reaction with chloride, bromide, and iodide ions. The test system was as follows: From 0.01 to 0.05 milliequivalent of the halogen compound (sodium salt) was placed in colorimeter cuvettes. Water was added to make a volume of 5 ml. and then 2 ml. of 50 per cent phosphoric acid were added. 1 ml. of a 1 per cent solution of the alkaloid (in 5 per cent phosphoric acid) was added, the solution was mixed and 0.5 ml. of a 10 per cent solution of potassium persulfate was added. The tubes were placed in a boiling water bath for 30 minutes, allowed to cool at room temperature for 30 minutes, and diluted to the original volume. The amount of color development was measured at 540 m $\mu$  in a Coleman spectrophotometer, model 6-A. The results with brucine, strychnine, and cinchonidine are summarized in Table I.

From the results given in Table I it is seen that chloride cannot be determined in the presence of bromide or iodide with brucine alone. It is possible, barring interference, to determine chloride in solutions containing no bromide or iodide, to determine bromide in the presence of chloride and in the absence of iodides, and to determine iodides in the presence of chlorides and bromides.

*Chloride Method*—The method is essentially that given above, a solution of brucine is used but the period of heating is extended to 1 hour. The usable range is from about 0.005 to 0.05 milliequivalent (or from 0.1 to 2.0 mg.) of chloride ion in 5 ml. of sample. With suitable modification and small colorimeter cuvettes, the sensitivity can be increased to 0.01 to 0.1

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mg in 1 ml of sample In Fig 1 the absorption curve as determined with the Beckman model DU spectrophotometer is given The wave-length of maximum absorption is in the range of  $490\text{ m}\mu$   $540\text{ m}\mu$  has been found to be a more useful wave-length for measurement As shown in Fig 2, Beer's law is obeyed in the specified range Theoretical recoveries of

TABLE I

*Approximate Ratios of Color Development of Halogens with Different Alkaloids*

Samples of 0.01 milliequivalent of the sodium salts were compared, as described in the text

Halide	Brucine	Strychnine	Cinchonidine
Chloride	1	0	0
Bromide	5	1	0
Iodide	8	1	1

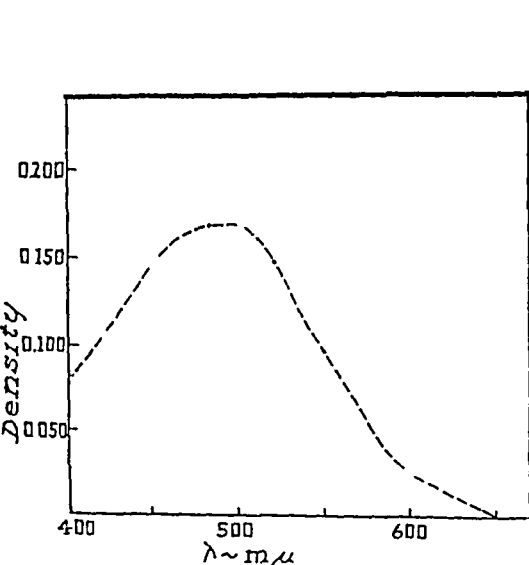


FIG 1

FIG 1 Absorption spectrum of the color developed in the reaction of 0.004 milliequivalent of chloride ion with brucine and persulfate

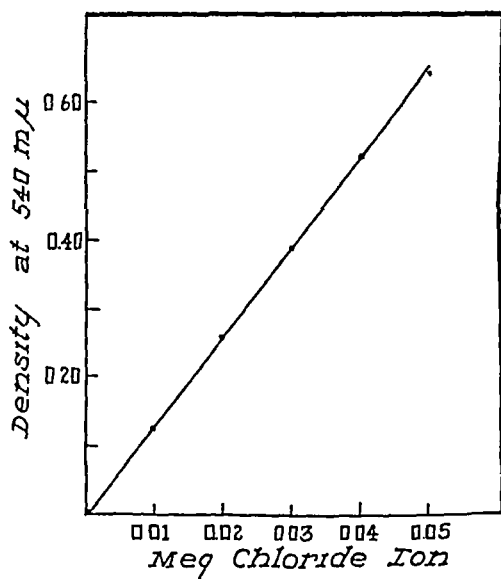


FIG 2

FIG 2 Dependence of color development upon concentration of chloride ion

chloride were found for cysteine, arginine, lysine, and histidine hydrochlorides Other halogens were the only important sources of interference encountered

*Determination of Chloride in Plasma or Serum*—The method for the determination of chloride has been adapted to the determination of chloride in plasma and serum in the absence of bromides 2 ml of an ordinary

1 10 tungstate or trichloroacetic acid filtrate were used. The protein precipitant was added to the standards to compensate for contamination with chloride. The colorimetric method was compared with the titrimetric method of Schales and Schales<sup>1</sup>. The colorimetric method proved to be more reproducible than the titrimetric method. In general, however, the colorimetric values were about 2 milliequivalents per liter higher than by the titrimetric procedure. In a series of 100 determinations covering all types of conditions met in the routine laboratory, the colorimetric procedure averaged 1.6 milliequivalents per liter higher than the titrimetric procedure. The details of the clinical application of the method will be presented elsewhere.

*Bromide Method*—If strychnine is used as a color reagent, bromide may be determined in the presence of chloride. In the absence of chlorides, the method in which brucine is used as a color reagent is more sensitive. When

TABLE II  
*Recovery of Bromide in Presence of Chloride*

Chloride was present in amounts equivalent to 100 milliequivalents of chloride per liter of plasma

Bromide added	Bromide recovered	Recovery
<i>m.eq</i>	<i>m.eq</i>	<i>per cent</i>
0.010	0.009	90
0.020	0.019	95
0.030	0.031	103
0.050	0.055	110

strychnine is used, a shorter period of heating (15 minutes) is desirable. The sensitivity is of the same order as for chloride with brucine as the reagent. Little opportunity has been found to apply the method to biological materials but recovery experiments have indicated that bromide in concentrations of from 25 to 200 milliequivalents per liter of plasma may be accurately estimated. In Table II, recoveries of bromide in the presence of chloride are illustrated. These samples were compared with standards containing no added chloride. If chloride was added to the standards to approximate the chloride concentration of plasma, theoretical recoveries were obtained.

#### SUMMARY

Colorimetric methods for chloride and bromide ions have been described. The method for chlorides has been applied to the determination of chloride in plasma in the absence of bromide.

<sup>1</sup> Schales, O., and Schales, S. S., *J. Biol. Chem.*, 140, 879 (1941)



# COLORIMETRIC ESTIMATION OF CYSTEINE\*

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(Received for publication, December 9, 1947)

In the preceding paper (1), the application of certain reactions of alkaloïds to the determination of chloride and bromide ions was described. It was found that in the presence of cysteine, in a similar system, but at lower temperatures, the reaction was modified so that a blue color was produced. The production of the blue color was proportional to the concentration of cysteine for a rather wide range of concentrations. In the presence of a high concentration of phosphoric acid, color formation with cysteine was repressed and, therefore, sulfuric acid was used. Acid concentration was found not to be a critical factor as long as uniform conditions were maintained. Since the color produced by cysteine was rapidly destroyed at the temperature of a boiling water bath, cysteine did not interfere with the determination of halogens.

The specificity of the method appeared to be somewhat greater than that of the reaction of Sullivan and Hess (2). As yet, no extraneous compounds or derivatives of cysteine have been found to react.

## EXPERIMENTAL

*Determination of Cysteine in Pure Solution*—To amounts of cysteine varying from 0.0005 to 0.005 milliequivalent in 5 ml. of solution were added 0.5 ml. of 50 per cent sulfuric acid, 2 ml. of 1 per cent brucine in 5 per cent sulfuric acid, 0.5 ml. of a 10 per cent solution of glycine, and 0.5 ml. of a 1 per cent solution of potassium persulfate. The development of color was allowed to proceed in a water bath adjusted to 30° for 30 minutes and was estimated at 660 m $\mu$  in a Coleman spectrophotometer, model 6-A. The data obtained are summarized in Fig. 1. The absorption spectrum of a sample was determined with a Beckman spectrophotometer (model DU) and is reproduced in Fig. 2. The wave-length 660 m $\mu$  was selected for routine measurements since, at this wave-length the absorption due to a reaction with halogens was negligible.

*Specificity of Reaction*—The following amino acids were tested and found not to influence the reaction when present in concentrations 10 times greater

\* These studies were supported by a grant from the United States Public Health Service.



than that of the cysteine alanine, glycine, valine, leucine, histidine, lysine, tryptophan, serine, threonine, proline, hydroxyproline, glutamic acid, aspartic acid, arginine, tyrosine, phenylalanine, and thyroxine. The list of sulfur-containing compounds which were negative includes glutathione, cysteinylglycine, homocysteine, methionine, S-benzylcysteine, N-benzoylcysteine, N-acetylcysteine, thioglycolic acid, hydrogen sulfide, S-methylcysteine, cystine, and homocystine. Glucose, purines, and pyrimidines were not found to interfere. The method was found to be unsuitable in

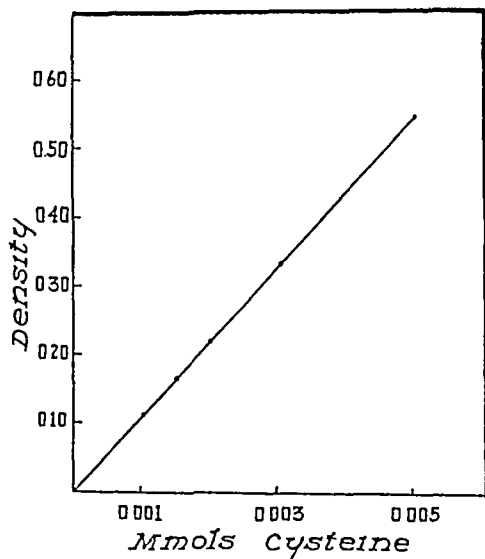


FIG 1

FIG 1 Dependence of optical density upon concentration of cysteine. The measurements were made in a Coleman junior spectrophotometer, model 6-A, at 660  $m\mu$ .

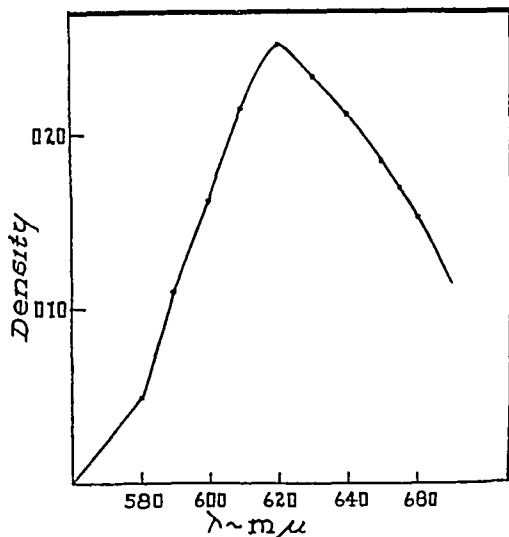


FIG 2

FIG 2 Absorption spectrum of the reaction of cysteine with brucine and persulfate. The measurements were made in a Beckman model DU spectrophotometer with a solution containing 0.001  $m$  of cysteine. The results are corrected for the absorption of a reagent blank.

the presence of ascorbic acid, epinephrine, thiourea, and heavy metals such as mercury and lead, all of which interfered with color development.

The method of Sullivan and Hess (2) was compared with the proposed method. The reactions with cysteine and characteristic derivatives of cysteine are summarized in Table I.

It is apparent that the reaction of Sullivan is positive for carboxyl-substituted derivatives of cysteine, whereas the proposed method is positive only with the unsubstituted cysteine molecule. Similar observations on the method of Sullivan and Hess have been made by White (3).

*Determination of Cystine in Pure Solution*—After reduction with metallic zinc, but not after treatment with sodium amalgam (since heavy metals interfere), cystine was determined either alone or in the presence of cysteine. To 10 ml of the solution containing between 0.0001 and 0.001 milliequivalent of cystine per ml in 0.1 N HCl were added 50 mg of zinc dust. The mixture was placed in a boiling water bath for 30 minutes, cooled to room temperature, and diluted to the original volume. The determination from this point was identical with the determination of cysteine. If a sample treated with zinc and an untreated sample were used, it was possible to

TABLE I

*Reactions of Method of Sullivan and Hess and Proposed Method with Cysteine and Its Derivatives*

Method	Cysteine	Cysteinyl glycine	S-Benzyl cysteine	N-Benzoyl cysteine
Sullivan	+	+	—	—
Proposed	+	—	—	—

TABLE II

*Recovery of Cystine and Cysteine in Mixtures*

The mixture was composed of equal parts of half cystine and cysteine, cysteine was used as a standard

Amount added	Cystine after Zn treatment	Cystine + cysteine	Cystine + cysteine after Zn treatment
mM	mM	mM	mM
0.00100	0.00105	0.00048	0.00101
0.00200	0.00209	0.00102	0.00208
0.00250	0.00246	0.00123	0.00256
0.00300	0.00301	0.00151	0.00307

estimate cysteine and cystine in the same solution. In Table II sample recoveries with solutions and mixtures of cysteine and cystine are given.

The method has been applied to the estimation of cystine and cysteine in hydrolysates of proteins. Theoretical values have been found in the recovery of cysteine or cystine added to hydrolysates prepared with sulfuric acid; these results, together with a comparison of the proposed method with other methods for cystine and cysteine in the analysis of proteins, will be presented at a later time.

An application of the method to the determination of cysteine in enzymatic digests is described in the following publication (4).

## SUMMARY

A new colorimetric method for cysteine has been described. It appears to be a more specific method than those previously described.

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# METABOLISM OF GLUTATHIONE ✓

## I HYDROLYSIS BY TISSUES OF THE RAT\*

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Glutathione is an obvious model for the study of the enzymatic synthesis of peptide bonds and, in addition, very little is known of the functions of glutathione in the organism. It is, therefore, of some interest to study the hydrolysis of this compound by enzymes of animal tissues. Previous studies of this problem (1-3) have been handicapped by the lack of adequate methods for the identification of the products of the hydrolysis. The method for cysteine, described in the preceding report (4), together with the method of Sullivan and Hess (5) for the determination of cysteine plus cysteinylglycine has been found to provide adequate means for studying the hydrolysis of glutathione by tissues of the rat. Amino-substituted derivatives of cysteine, such as  $\gamma$ -glutamylcysteine, are not measured by either method. Schroeder and Woodward (1) have reported earlier that  $\gamma$ -glutamylcysteine does not react in the method of Sullivan.

### EXPERIMENTAL

*Methods of Analysis*—Since cysteinylglycine plus cysteine is measured by the Sullivan and Hess method and only cysteine by that of Nakamura and Binkley, it was possible to determine cysteinylglycine as the difference between the two methods. In Fig 1 the absorption curves of the reaction of cysteine and of cysteinylglycine with the Sullivan reagents are given.<sup>1</sup> It is seen that at 540  $m\mu$  the absorption of cysteinylglycine was identical with that of cysteine. Hence, readings taken at 540  $m\mu$  were a measure of cysteine plus cysteinylglycine.

An alternative method of measurement of cysteinylglycine was apparent from the characteristics of the absorption curves in Fig 1. When two arbitrary wave-lengths for measurement, one on each side of 540  $m\mu$ , were taken (500 and 580  $m\mu$  were selected), it was possible to determine cysteinylglycine.

\* These studies were supported by a grant from the United States Public Health Service.

<sup>1</sup> The measurements reported here were made with a Coleman junior spectrophotometer, the same instrument that was used in the analysis of the digests. The results are illustrative only and are not to be construed as a determination of absorption curves of the Sullivan reaction as determined with a precision instrument.

glycine and cysteine in the presence of each other by the Sullivan method alone. The ratios of the optical density at 500  $m\mu$  to that at 580  $m\mu$  were determined for a wide range of mixtures of cysteinylglycine and cysteine, a straight line relationship was obtained (Fig 2). From measurements of optical density made at 500, 540, and 580  $m\mu$ , it was possible to calculate the concentration of cysteine and cysteinylglycine in one sample. From the measurement at 540  $m\mu$ , the total of cysteine plus cysteinylglycine was obtained from a calibration chart prepared with known amounts of cysteine. From the ratio of the density at 500 to that at 580  $m\mu$ , the percentage of cysteine was obtained from a chart similar to Fig 2.

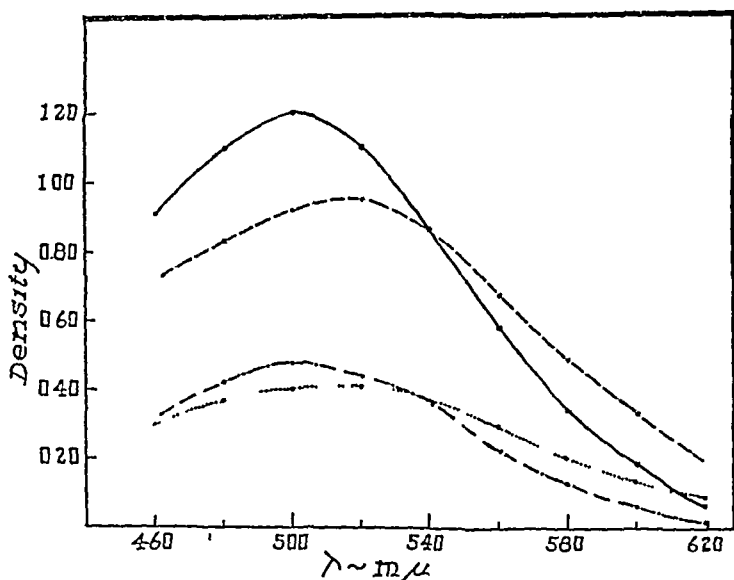


FIG 1 Absorption curve of the Sullivan reaction with cysteine and cysteinylglycine. The upper lines are for a concentration of  $5 \times 10^{-4}$  M of each substance and the lower lines are for a concentration of  $2 \times 10^{-4}$  M of each substance. The solid line and that of the upper curve in the lower part are absorption curves of cysteinylglycine.

All analyses were made on 2 ml of a trichloroacetic acid filtrate. The concentration of the trichloroacetic acid in the filtrate was 5 per cent. It was necessary, for the Sullivan method for cysteinylglycine, to prepare a chart similar to Fig 2 but containing, in each determination, the amount of trichloroacetic acid found in the filtrates. The two methods for cysteinylglycine gave good agreement throughout these studies, the results reported here as cysteinylglycine are those determined as the difference between the methods of Nakamura and Binkley and of Sullivan and Hess. Cysteine values are those of the method of Nakamura and Binkley.<sup>2</sup>

<sup>2</sup> If glutathione, in a solution of 5 per cent trichloroacetic acid as in the filtrates, was autoclaved at 20 pounds for 30 minutes, an almost quantitative release of cysteine was obtained.

*Preparation of Homogenates and Extracts*—Tissues, from rats killed by decapitation, were ground immediately with 10 volumes of saline in a glass tissue grinder (Scientific Glass Apparatus Company). This homogenate was used without further treatment in those instances designated as homogenate. To prepare an extract, the homogenate was centrifuged for approximately 15 minutes at 3000 R P M. The supernatant was used in those experiments designated as extract.

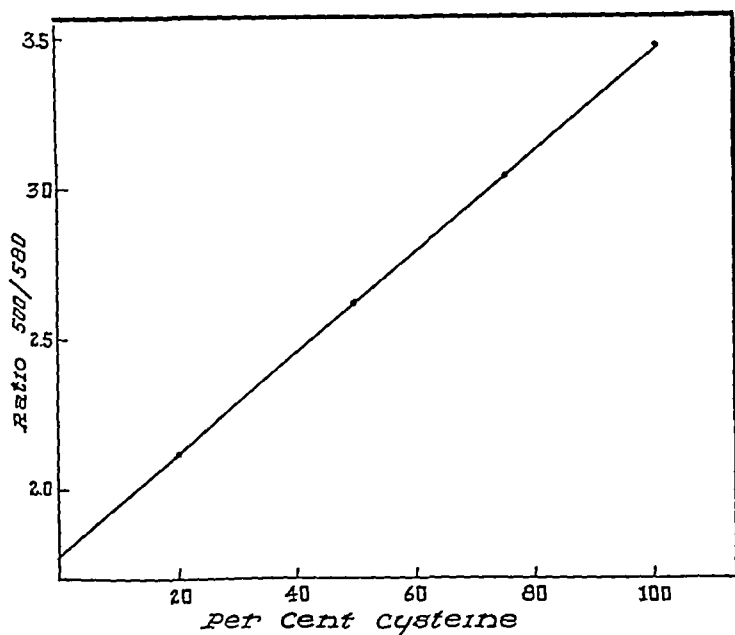


FIG. 2. Relation of percentage of cysteine to the 500/580  $m\mu$  ratio. Various mixtures of cysteine and cysteinylglycine were prepared and the ratios of density at 500  $m\mu$  to that at 580  $m\mu$  of the Sullivan reaction of the mixtures were determined.

In the preparation of the dialyzed extract, the material was placed in Visking cellulose casing and allowed to remain in running tap water for 2 hours. In certain experiments the dialysis was against distilled water at 4° for 24 hours.

The following routine was followed for all work reported here. To 5 ml

teinyglycine was obtained. No cysteine was formed. Under similar conditions,  $\gamma$ -glutamylcysteine should be decomposed to cysteine. This method was used for the detection of  $\gamma$ -glutamylcysteine. No indication of the presence of  $\gamma$ -glutamylcysteine was obtained in the analysis of filtrates from the hydrolysis of glutathione by enzymes of the kidney of the rat. It will be desirable, however, to check results by the method with authentic samples of  $\gamma$ -glutamylcysteine.

of 0.1 M buffer were added 2 ml of glutathione (5 mg per ml except in the substrate concentration studies), 2 ml of enzyme preparation, and water to make a total of 10 ml. Metal ions and other materials were dissolved in water and added in lieu of water. All solutions were saturated with nitrogen and the digestions were at 30° in stoppered tubes under an atmosphere of nitrogen. 4 ml of the digestion mixture were taken for each analysis and were added to a mixture of 5 ml of water and 1 ml of 50 per cent trichloroacetic acid. The filtrate was used immediately in the analyses, it was found unnecessary to reduce before the determinations. The buffer called "barbiturate" was made from diethyl barbiturate (veronal).

*Activity of Different Tissues*—Only the kidney of the rat was found to contain enzymes capable of cleaving glutathione to cysteinylglycine and

TABLE I

*Hydrolysis of Glutathione by Different Tissues*

Homogenates of the tissues, prepared as described in the text, were tested in barbiturate buffer, pH 8.0. The time of incubation was 90 minutes at 30°.

Tissue		Cysteine produced	Cysteinylglycine produced
Rat	Kidney	+	+
	Liver	—	—
	Spleen	—	—
	Heart	—	—
	Skeletal muscle	—	—
	Testicles	—	—
	Thymus	—	—
Pig	Kidney (desiccated)	—	+
	Pancreas (desiccated)	—	+
	Duodenum "	—	—

cysteine. Liver, spleen, muscle, heart, testicles, and thymus were found to have no activity (Table I). Desiccated pancreas, but not desiccated duodenum, of the pig contained an enzyme capable of cleaving glutathione to cysteinylglycine. Grassmann *et al.* (6) have reported that oxidized glutathione is hydrolyzed to glycine by carboxypeptidase of pancreas.

*Comparison of Different Preparations of Rat Kidney*—Homogenates, filtrates, and dialyzed preparations from the same kidneys were compared. The results are given in Table II. Considerable activity is lost by centrifugation and a further small loss is found with dialysis.

*Effect of Heating Enzyme Preparation*—Samples of dialyzed extract and crude extract were heated at 40°, 50°, 60°, and 70° for 15 minutes, cooled to room temperature, centrifuged to remove flocculated protein, and tested for activity. In Table III the results are given. It is apparent that the

activity of the preparation toward cysteinylglycine is more or less selectively destroyed by heating. The sensitivity to heat is greater with the dialyzed preparation.

*pH-Activity Relationship of Dialyzed Material*—Activity of the dialyzed preparation was tested at different pH values, from 4.8 to 8.3. At lower

TABLE II

*Comparison of Different Preparations of Kidney Tissue*

The different preparations were tested in barbiturate buffer, pH 8.2. Incubation was for 90 minutes at 30°.  $310 \times 10^{-4}$  mm of glutathione in each digest.

Preparation	Cysteine produced	Cysteine + cysteinylglycine produced
	$\text{mm} \times 10^{-4}$	$\text{mm} \times 10^{-4}$
Homogenate	168	243
Filtrate	60	212
Dialyzed filtrate	48	187
“ “ heated at 50°	36	137

TABLE III

*Heat Inactivation Studies*

The preparation was heated for 15 minutes at the indicated temperature and tested in barbiturate buffer, pH 8.2. The incubation was for 30 minutes at 30°.  $310 \times 10^{-4}$  mm of glutathione present.

Preparation	Temperature of heating	Cysteine produced	Cysteinylglycine produced
	C	$\text{mm} \times 10^{-4}$	$\text{mm} \times 10^{-4}$
Dialyzed extract	Control	50	169
“ “	40	49	130
“ “	50	17	104
“ “	60	0	0
“ “	70	0	0
Crude extract	Control	98	182
“ “	40	90	176
“ “	50	66	169
“ “	60	18	94
“ “	70	0	0

values of pH, the ability to produce cysteine was decreased. The optimum conditions for the production of cysteine appeared to be well on the alkaline side, toward pH values of 8.0 (Table IV).

*Comparison of Phosphate and Barbiturate Buffers*—In the experiments on the activity of the dialyzed preparation at different pH values, the buffers employed were acetate, phosphate, and barbiturate. At values near pH



8.0, the activity, in the presence of phosphate buffer, was much greater than with barbiturate buffer (Table V). This apparent activation by phosphate is probably due to the removal of calcium ions. As is shown in Table V, calcium ions are inhibitory, the inhibition can be removed by the addition of phosphate or citrate.

*Effects of Various Metallic Ions*—The dialyzed preparation was utilized in a study of possible metal activation (Table VI). In confirmation of the

TABLE IV  
*pH Activity Curve of Dialyzed Extract*

0.1 M buffers were used throughout. The time of incubation was 30 minutes at 30°.  $310 \times 10^{-4}$  mM of glutathione present.

pH	Buffer	Cysteine produced	Cysteine + cysteinyl glycine produced
		mM $\times 10^{-4}$	mM $\times 10^{-4}$
4.8	Acetate	0	63
6.5	Phosphate	33	63
7.0	"	49	84
7.5	"	62	105
8.0	Barbiturate	29	105

TABLE V

*Inhibitory Effect of Calcium and Effect of Phosphate Buffer and Citrate*

All digests were in 0.05 M buffer, pH 8.0, with an extract of kidney tissue. The time of incubation was 90 minutes at 30°.  $310 \times 10^{-4}$  mM of glutathione present.

Experiment No.	Digest	Cysteine produced	Cysteine + cysteinyl glycine produced
		mM $\times 10^{-4}$	mM $\times 10^{-4}$
I	Barbiturate buffer	125	187
	Phosphate buffer	162	212
II	Barbiturate buffer	162	200
	" " + 0.01 M $\text{CaCl}_2$	75	162
	" " + 0.01 " Na citrate	200	200

above experiments, calcium was found to inhibit the enzyme producing cysteine. Magnesium behaved like calcium, cobalt apparently inhibited both enzymes.

*Other Occurrences of Enzyme Hydrolyzing Cysteinylglycine*—If the dialyzed preparation is heated at 50° for 15 minutes, most, if not all, of the activity toward cysteinylglycine is destroyed. The addition of an extract of liver tissue restores the activity. The restoration is abolished by heating the liver extract at 70° for 15 minutes (Table VII). Similar results were ob-

tained with extracts of muscle. The enzyme hydrolyzing cysteinylglycine is, therefore, of wider occurrence than the one hydrolyzing glutathione to cysteinylglycine.

TABLE VI  
*Effects of Metal Ions*

Incubation was for 30 minutes with a dialyzed extract at 30° in 0.05 M barbiturate buffer at pH 8.2.  $310 \times 10^{-4}$  mM of glutathione present. Metallic ions were added, as chlorides, to make a final concentration of 0.001 M.

Metallic ion	Cysteine produced	Cysteine + cysteinylglycine produced
	mM $\times 10^{-4}$	mM $\times 10^{-4}$
Control	66	126
Mg <sup>++</sup>	33	126
Co <sup>++</sup>	25	84
Mn <sup>++</sup>	68	112
Ca <sup>++</sup>	0	126

TABLE VII  
*Effect of Extract of Liver on Glutathione Hydrolysis*

Dialyzed extracts of kidney and liver tissue were used. 1 ml. of liver extract and 1 ml. of kidney extract, in a total volume of 10 ml., were tested as indicated in 0.05 M barbiturate buffer, pH 8.2. The time of incubation was 90 minutes at 30°.  $310 \times 10^{-4}$  mM of glutathione present. "Heated" preparations were extracts heated at the temperatures indicated for 15 minutes.

Enzyme preparation	Cysteine produced	Cysteine — cysteinylglycine produced
	mM $\times 10^{-4}$	mM $\times 10^{-4}$
Liver extract	0	0
Kidney "	19	245
Liver " + kidney extract	143	236
Heated kidney extract (50°)	0	238
" " " (50°) + liver extract	130	286
" " " (50°) + heated liver extract		
(70°) -	0	287

*Consideration of Kinetics of Hydrolysis*—In Figs. 3, 4, and 5 certain aspects of the kinetics of the hydrolysis of glutathione to cysteinylglycine and cysteine are summarized. The data, summarized in Figs. 3 and 4, were characteristic of consecutive reactions. The production of cysteine was delayed until the concentration of cysteinylglycine reached appreciable levels. The concentration of cysteinylglycine was found to reach a constant value. The experiments reported in Figs. 3 and 4 were with the same

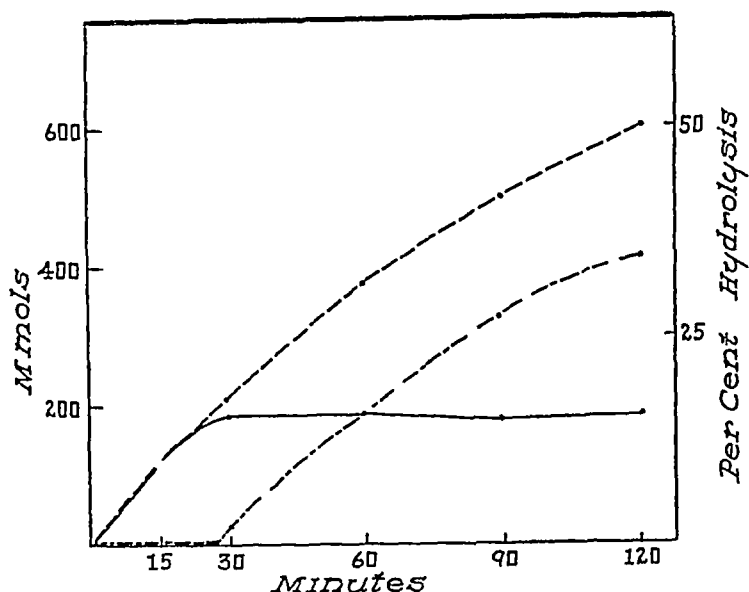


FIG 3 Hydrolysis of glutathione  $1200 \times 10^{-4}$  mm of glutathione was incubated with a dialyzed extract of kidney tissue in 0.05 M barbiturate buffer, pH 8.2. The upper broken line represents the production of cysteinylglycine plus cysteine, the production of cysteine is represented by the lower broken line, and cysteinylglycine production is represented by the solid line.

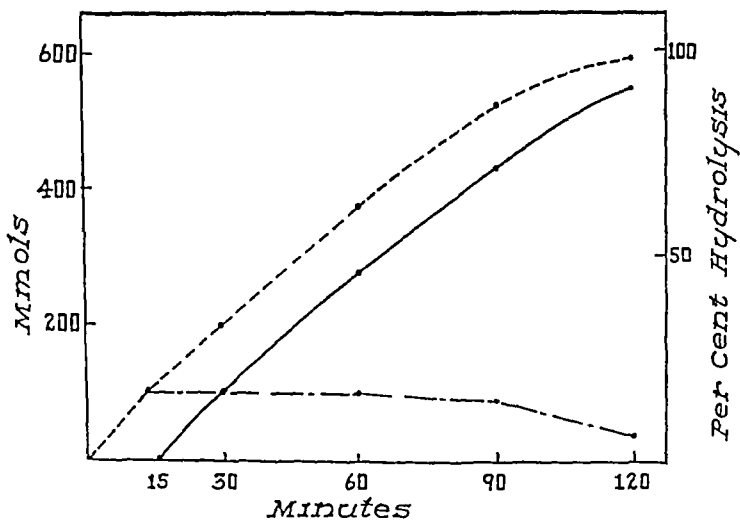


FIG 4 Hydrolysis of glutathione  $600 \times 10^{-4}$  mm of glutathione was incubated with a dialyzed extract of kidney tissue in 0.05 M barbiturate buffer, pH 8.2. The upper line represents the production of cysteinylglycine plus cysteine, the production of cysteine is represented by the solid line. The lowest line represents the production of cysteinylglycine.

extract. With the greater amount of substrate (Fig 3), the concentration of cysteinylglycine remained constant for the greater part of the hydrolysis.

With less substrate (Fig 4) hydrolysis was practically complete and, as would be expected, the concentration of cysteinylglycine decreased sharply as the hydrolysis neared completion. A comparison of Figs 3 and 4 is interesting. It is apparent that higher concentrations of glutathione are inhibitory in so far as the formation of cysteine is concerned.

The data, summarized in Fig 5, relating the velocity of the hydrolysis of glutathione to cysteinylglycine to the concentration of the substrate, glutathione, are characteristic of hydrolytic enzymes. At low concentrations, the reaction was found to follow first order kinetics, changing to zero order kinetics at higher concentrations.

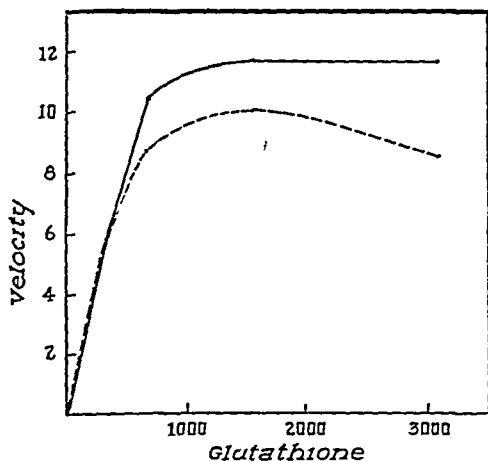


Fig 5 Velocity substrate concentration relationships. Only the values for cysteine plus cysteinylglycine are recorded. Incubation was in 0.05 M barbiturate buffer with a dialyzed extract of kidney tissue. Velocity is expressed in  $\text{mm} \times 10^{-4}$  per minute and glutathione concentration is in  $\text{mm} \times 10^{-4}$  per ml of enzyme in a total volume of 10 ml. The broken line represents the results with the dialyzed extract. The solid line represents the dialyzed extract with 0.001 M  $\text{MgCl}_2$ .

The only valid indication for the participation of an inorganic ion was obtained from these studies. Previous studies (Table VI) with the substrate in low concentration, were found to give equivocal results. When measurements were made with higher concentration of substrate, it was found that at levels of substrate near or greater than saturation additional substrate was inhibitory. The inhibition by higher concentrations of substrate was largely abolished by the addition of magnesium ions. As is illustrated in Table VI, magnesium ions were found to be inhibitory in so far as the production of cysteine was concerned. The results in Fig 5 relate only to the production of cysteinylglycine, the production of cysteine was again found to be inhibited by the addition of magnesium ions.

## DISCUSSION

The results reported here are preliminary to a purification and detailed study of the enzymes responsible for the hydrolysis of glutathione. The evidence is compatible with the concept that the hydrolysis of glutathione to cysteine is a two-step process involving the intermediary formation of cysteinylglycine. In many experiments, quantitative formation of cysteinylglycine was indicated. There was little reason, therefore, to assume that appreciable amounts of  $\gamma$ -glutamylcysteine were formed. No  $\gamma$ -glutamylcysteine was detected by a method assumed to be of suitable specificity and sensitivity.

The enzyme responsible for the hydrolysis of glutathione to cysteinylglycine was found to be more stable to heat and to be active at lower values of pH than the enzyme responsible for the hydrolysis of cysteinylglycine. In so far as intracellular enzymes are concerned, the enzyme responsible for the hydrolysis of glutathione to cysteinylglycine is, in the rat, limited to the kidney. The significance of this limited distribution is not apparent.

The enzyme responsible for the hydrolysis of cysteinylglycine was found in muscle and liver as well as in the kidney and probably occurs in all tissues. Studies are under way, with cysteinylglycine as the substrate, to determine whether this enzyme is identical with any of the previously characterized peptidases of tissues.

Little, if any, clear cut evidence was found for the occurrence of a dialyzable component of the enzyme system. These results are in sharp disagreement with those of Neubeck and Smythe (3), these workers utilized the liver of the guinea pig as the source of their enzyme and did not distinguish the production of cysteine from that of cysteinylglycine.

Even though the results of Schroeder and Woodward (1) and of Woodward and Reinhart (2) are not valid in so far as the production of cysteine is concerned, their results from following the formation of glutamic acid and pyrrolidonecarboxylic acid are in general agreement with our conclusions that the first step in the hydrolysis of glutathione by kidney tissue is the cleavage at the  $\gamma$ -glutamylcysteine linkage.<sup>3</sup>

## SUMMARY

The hydrolysis of glutathione by enzymes found in the kidney of the rat has been followed by a combination of methods for the determination of cysteine and cysteinylglycine. The observations were found to be com-

- It has since been found that desiccated kidney tissue of the hog (VioBin Corporation) does not contain the enzyme responsible for the release of cysteine but does contain appreciable amounts of the enzyme responsible for the production of cysteinylglycine. A marked activation with magnesium ions has been observed with this preparation.

patible with the concept that the hydrolysis to cysteine is a two-step process with the intermediary formation of cysteinylglycine. The enzyme responsible for the cleavage of glutathione to cysteinylglycine is, in the rat, limited to the kidney. The enzyme responsible for the cleavage of cysteinylglycine to cysteine is of much wider distribution.

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## LETTERS TO THE EDITORS

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### $\alpha$ -AMINOADIPIC ACID A PRODUCT OF LYSINE METABOLISM\*†

Sirs

As part of a study of protein and peptide metabolism lysine was synthesized with  $C^{14}$  in the  $\epsilon$  position and resolved into the L and D isomers. 10 mg of labeled lysine dihydrochloride (either L- or D-) and 0.66 gm (wet weight) of guinea pig liver homogenate were added to a reaction mixture containing 1.3 per cent of an amino acid mixture corresponding to the composition of casein except for lysine and 0.01 M  $\alpha$ -ketoglutarate, all in a final volume of 4 ml of isotonic saline solution.<sup>1</sup> The reaction was carried out under oxygen for 6 hours at 38°.

After precipitation of the proteins by boiling at pH 5.0, a fraction of the non-protein filtrate was chromatographed on filter paper.<sup>2</sup> Ninhydrin gave a lysine spot which was radioactive and another radioactive spot in the glutamic acid region. This indicated the presence of a dicarboxylic  $\alpha$ -amino acid different from glutamic acid, since to obtain glutamic acid from the labeled lysine would entail removing the radioactive  $\epsilon$ -carbon.

The chromatographic behavior of this radioactive substance on filter paper and on Lloyd's reagent suggested, in view of the source of the  $C^{14}$ , that the unknown substance might be  $\alpha$ -aminoadipic acid. Accordingly the latter was synthesized, it gave the same filter paper chromatogram as the unknown.

Radioactive  $\alpha$ -aminoadipic acid (probably mixed with glutamic acid) was isolated from the reaction mixture as a barium salt by the following procedure. After hydrolysis of the non-protein filtrate with hydrochloric acid and chromatography on Lloyd's reagent,<sup>3</sup> whereby the hexone bases were removed, the non-basic amino acid fraction was precipitated with barium from 75 per cent ethanol. The barium was removed from the precipitate, and the dicarboxylic acids again precipitated with barium. 99 per cent of the radioactivity originally present in the fraction was found in

\* This work is a part of that done under contract with the Office of Naval Research, United States Navy Department.

† The  $C^{14}$  used in this investigation was supplied by the Monsanto Chemical Company, Clinton Laboratories, and obtained on allocation from the United States Atomic Energy Commission.

<sup>1</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **171**, 363 (1947).

<sup>2</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

<sup>3</sup> Bergdoll, M. S., and Doty, D. M., *Ind. and Eng. Chem.*, **18**, 600 (1946).



this barium precipitate The barium was again removed, and a fraction obtained by crystallization from 20 per cent hydrochloric acid was washed, dried, and then chromatographed on filter paper One strong spot was obtained with ninhydrin in the same position as that of the original unknown, it contained all the estimated amount of radioactivity Two other very faint spots were obtained, one of which corresponded to aspartic acid, the other to alanine or threonine, neither had radioactivity There was no lysine spot, nor was there any radioactivity in the region of the lysine spot

The above evidence is conclusive, short of isolation of the compound, that lysine is deaminated in the  $\epsilon$  position to  $\alpha$ -aminoadipic acid

The following evidence showed that the process is an enzymatic one and added to the proof that the radioactivity did not come from contamination by the labeled lysine originally added The dicarboxylic acid fraction contained no radioactivity when the homogenate was first boiled, much more radioactivity was found when the reaction was carried out at pH 7.5 than at 8.2 and with the L than with the D form of lysine The activity found with the latter may be due entirely to the presence of a small amount (10 per cent or less) of the L form remaining after resolution

Under our best conditions so far 1  $\gamma$  of lysine is converted to  $\alpha$ -aminoadipic acid per 10 mg (dry weight) of guinea pig liver homogenate per hour

In an accompanying communication evidence is presented that lysine is converted to  $\alpha$ -aminoadipic acid in rat kidney slices Dr H. K. Mitchell kindly tested the ability of synthetic DL- $\alpha$ -aminoadipic acid to replace lysine in a *Neurospora lysineless* mutant It was able to do so <sup>4</sup>

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<sup>4</sup> Mitchell, H. K., and Houlahan, M. B., unpublished observation

# $\alpha$ -AMINOADIPIC ACID IN ARGININE FORMATION\*

Sirs

In a previous study we had shown that arginine may be synthesized by the transfer of the amino group of glutamic or aspartic acid to citrulline<sup>1</sup> Since lysine was also active, it was suggested at the time that it was first converted to glutamic acid which then aminated citrulline However, experiments in liver homogenate with lysine labeled in the  $\epsilon$  position with  $C^{14}$  showed that a radioactive dicarboxylic acid was formed This excluded glutamic acid formation by oxidative removal of the radioactive carbon  $\alpha$ -Aminoadipic acid has now been shown to be very probably the product of the reaction<sup>2</sup>  $\alpha$ -Aminoadipic acid has been shown to be active in transamination by Braunstein,<sup>3</sup> who interpreted our results with lysine as being due to its conversion to this dicarboxylic acid

Approximately 10 mg (fresh weight) of rat kidney slices in 4 ml of Krebs' bicarbonate Ringer's solution, 22 mg per cent of citrulline in each vessel Temperature, 38°, time, 1 hour

Metabolite	Increase in arginine, mg per gm fresh tissue per hr
DL-Glutamic acid (18.5 mg %)	1.9
DL- $\alpha$ -Aminoadipic acid (20 mg %)	0.9
DL-Lysine dihydrochloride (27.5 mg %)	0.3

The experiment presented in the table shows that  $\alpha$ -aminoadipic acid can aminate citrulline, and that the relative reaction rates are compatible with the hypothesis that lysine is converted into  $\alpha$ -aminoadipic acid

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<sup>1</sup> Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, **141**, 717 (1943)

<sup>2</sup> Borsook, H., Deasy, C. L., Haagen-Smit, A. J., Keighley, G., and Lowy, P. H., *J. Biol. Chem.*, **173**, 423 (1948)

<sup>3</sup> Braunstein, A. E., in Anson, M. L., and Edsall, J. T., *Advances in protein chemistry*, New York, **3**, 40 (1947)



# THE SPONTANEOUS CONVERSION OF CRYSTALLINE TETANAL TOXIN TO A FLOCCULATING ATOXIC DIMER\*

Sirs

It has been noted in this laboratory that crystalline tetanal toxin<sup>1</sup> upon standing at 0° in neutral isotonic salt solutions rapidly loses toxicity with no loss in its flocculating power with tetanal antitoxin. Dipolar ions such as glycine greatly retard this spontaneous detoxification. Tetanal toxin, recrystallized four times, is electrophoretically homogeneous and acts as a single substance in the flocculation reaction. In constant solubility determinations, at least 95 per cent of the material behaves as a solid phase consisting of one component. After standing for 10 days at 0°, 75 per cent of the toxicity was lost and the homogeneity of the solution as determined by constant solubility<sup>2</sup> was markedly altered. Fully 50 per cent of the material showed a decreased solubility in 1.4 M ammonium sulfate at pH 7 and the solution appeared to contain two molecular species. In the ultracentrifuge from 55 to 60 per cent of the molecules had a sedimentation constant of 7, and the remaining molecules sedimented at 4.5 S. Small samples of equal volumes were recovered from the top and bottom of the analytical cell. Immunological analysis of these samples revealed that the top fraction, containing only molecules sedimenting at 4.5 S, possessed 90 per cent of the total toxicity of the solution. The bottom fraction, consisting of both molecular species, contained less than 10 per cent toxin. Toxicity of this low magnitude in the bottom fraction was unexpected and studies are being undertaken to determine the cause. The bottom fraction contained at least 60 per cent of the total flocculating units of the solution, and flocculated readily with antitoxin at a Kf of 20 minutes when tested against 50 units of antitoxin. The remaining flocculating units were present in the "light" fraction and this material had a Kf of 10 minutes.

These results suggest that crystalline tetanal toxin spontaneously converts to a flocculating atoxic dimer. It is unknown whether the above phenomenon enters into the detoxification of tetanal toxin by formaldehyde. It has been postulated<sup>3</sup> that formaldehyde causes condensation of toxin molecules during detoxification. However, highly purified diphtherial toxin<sup>4</sup> (assuming that it had not converted to toxoid prior to analysis) and

\* Aided by a grant from Lederle Laboratories Division, American Cyanamid Company

<sup>1</sup> Pillemer, L., Wittler, R. G., and Grossberg, D. B., *Science*, 103, 615 (1946)

<sup>2</sup> Northrop, J. H., *J. Gen. Physiol.*, 25, 467 (1942)

<sup>3</sup> Eaton, M. D., *Bact. Rev.*, 2, 3 (1938)

<sup>4</sup> Pappenheimer, A. M., Jr., *J. Biol. Chem.*, 120, 543 (1937)

pure diphtherial toxoid<sup>5</sup> both have the same sedimentation constant of 4.6 S

Work is in progress to determine the mechanism of this reaction and its possible rôle in the formation of toxoids, and to ascertain whether this phenomenon is characteristic of tetanal toxin or whether it is common to all bacterial toxins

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<sup>5</sup> Pillemer, L., Toll, D., and Badger, S. J., *J. Biol. Chem.*, **170**, 571 (1947)

# GLYCINE AS A PRECURSOR OF PURINES IN YEAST

Sirs

It has been demonstrated by the work of Sonne, Buchanan, and Delluva<sup>1</sup> and of Shemin and Rittenberg<sup>2</sup> that glycine is utilized for uric acid synthesis in both pigeon and man, and occupies positions 4, 5, and 7. If all purine synthesis follows the same course, one would expect glycine to appear in the nucleic acid purines. The experiments reported here indicate that in yeast glycine is used for the synthesis of nucleic acid purines in the same manner as for uric acid synthesis in humans and pigeons.

**Yeast Growth**—To approximately 80 gm wet weight of actively growing *Torulopsis utilis* in 2 liters of modified Williams' solution<sup>3</sup> were added 374 mg of glycine containing 32 atom per cent excess N<sup>15</sup>, and growth was allowed to continue for 1 hour.

*Glycine N in Nucleic Acid Purines*

Compound	Atom per cent excess N <sup>15</sup>
Adenine	0.428
Guanine	0.490
Glycine (from guanine)	0.858

**Purine Extraction**—The yeast was fragmented by mechanical vibration with glass beads<sup>4</sup> and extracted with boiling alcohol-ether (3:1). After being dried *in vacuo*, 17.4 gm of dry yeast were obtained. This yeast powder was washed with cold 0.05 M of HCl and then dissolved in 0.1 M of NaOH. Trichloroacetic acid was added to 5 per cent, and the polynucleotides of the yeast were extracted and hydrolyzed by heating the solution to 90° as described by Schneider.<sup>5</sup> After being filtered, the solution was refluxed 1 hour with 1 M HCl, and then made alkaline with ammonia. The purines were precipitated with ammoniacal AgNO<sub>3</sub>. Following decomposition of the washed silver salts with hot dilute HCl, guanine was precipitated by adjusting the pH to 5.5 and purified by several reprecipitations. Adenine was precipitated from the mother liquor with  $\beta$ -naphthalenesulfonic acid and recrystallized from an alcohol-ether mixture.

**Degradation**—Glycine containing the nitrogen atom in position 7 was

<sup>1</sup> Sonne, J. C., Buchanan, J. M., and Delluva, A. M., *J. Biol. Chem.*, **166**, 395 (1946).

<sup>2</sup> Shemin, D., and Rittenberg, D., *J. Biol. Chem.*, **167**, 875 (1947).

<sup>3</sup> Sperber, E., *Ark. Kemi, Mineral o. Geol.*, **21**A, No. 3 (1945).

<sup>4</sup> Hammarsten, E., *Acta med. Scand.*, suppl. 196, 634 (1947).

<sup>5</sup> Schneider, W. C., *J. Biol. Chem.*, **161**, 293 (1945).

isolated from the guanine by the method of Wulff<sup>6</sup> 100 mg of guanine were heated in a sealed tube with 7 ml of concentrated HCl at 200° for 18 hours Glycine was separated as the *p*-toluenesulfonyl derivative After two recrystallizations from water, the melting point was 147–148°

The results of the mass spectrometer analyses are shown in the table If one assumes symmetrical distribution of N<sup>15</sup> in positions other than 7, the average atom per cent excess N<sup>15</sup> for nitrogen atoms 1, 3, and 9 will be 0.398 as compared with 0.858 for position 7 Thus glycine plays the same rôle in the synthesis of nucleic acid purines of yeast as it does in the formation of uric acid in the pigeon and in man

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<sup>6</sup> Wulff, C, *Z physiol Chem*, **17**, 473 (1893)

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# COZYMASE AS A PART OF THE HEPATIC ESTROGEN-INACTIVATING SYSTEM\*

Sirs

Although liver slices are able to inactivate natural estrogens,<sup>1</sup> liver mince has been found to be inefficient and unpredictable.<sup>2</sup> Similarly, almost all preparations of rat liver mince tested in this laboratory inactivated  $\alpha$ -estradiol only to a slight degree in comparison with slices prepared from the identical livers. The best of the active minces only approached the activity of the least active preparation of slices.

No. of assay rats	$\alpha$ Estradiol	Liver slices	Liver mince	Supernatant	Nicotin amide	Cozymase*	Uterine weight, average
	$\gamma$	mg	mg	cc	mg	mg	mg
25	0	0	0	0	0	0	22.4
8	0	50	0	0	0	0	19.8
38	0	0	50	0	0	0	22.1
14	0.3	0	0	0	0	0	87.5
27	0.3	50	0	0	0	0	35.4
24	0.3	0	50	0	0	0	74.8
12	0.3	0	50	0	1.5	0	31.7
13	0.3	0	50	1.0	0	0	39.4
15	0.3	0	50	0	0	0.15†	29.8
8	0.3	0	50	0	0	0.15‡	68.4

\* Containing 60 per cent diphosphopyridine nucleotide

† Cozymase mixed with  $\alpha$ -estradiol and then added to the mince

‡ Cozymase added 30 minutes before the  $\alpha$ -estradiol

This suggested that one of the participating materials is unstable in a broken cell preparation and might be enzyme-labile. Accordingly, livers were sliced into hot physiologic saline, then boiled for 10 minutes, and the supernatant added to a mince preparation. When enough of this supernatant was added to liver mince, in each instance the mince inactivated  $\alpha$ -estradiol to the same extent as did the slices from the same liver. It was suspected that cozymase (diphosphopyridine nucleotide) was the heat-stable component in the supernatant, since cozymase is rapidly inactivated in liver mince.<sup>3</sup> Recent evidence indicates that cozymase is involved in the

\* This work was done under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

<sup>1</sup> Heller, C. G., *Endocrinology*, 26, 619 (1940). Twombly, G. H., and Taylor, H. C., Jr., *Cancer Res.*, 2, 811 (1942).

<sup>2</sup> Zondek, B., *Genital functions and their hormonal regulation*, Baltimore (1941).

<sup>3</sup> Mann, P. J. G., and Quastel, J. H., *Biochem. J.*, 35, 502 (1941). Handler, P., and Klein, J. R., *J. Biol. Chem.*, 143, 49 (1942).



hepatic inactivation of testosterone<sup>4</sup> A commercial preparation of cozymase which was said to be free of coenzyme II and flavin-adenine-dinucleotide (Schwarz Laboratories, Inc, lot CO-4704) and of 60 per cent purity was tested When this cozymase was added to liver mince 30 minutes before  $\alpha$ -estradiol, the mixture failed to inactivate the estrogen Cozymase added to this mince simultaneously with the estrogen enabled it to inactivate the  $\alpha$ -estradiol as well as the slices from the same liver Mince prepared from fresh liver in a solution of 0.1 per cent nicotinamide (which protects cozymase) was also as active as slices from the same liver

The accompanying table gives average values from several experiments The materials were incubated by shaking in a water bath at 37.5° for 1 hour and tested for estrogenic activity according to the method of Lauson *et al*<sup>5</sup> The results are expressed as the average uterine weight of the recipient rats

It therefore appears that the heat-stable component of the hepatic enzyme system for estrogen inactivation is cozymase

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<sup>4</sup> Sweat, M. L., and Samuels, L. T., *J Biol Chem*, **173**, 433 (1948)

<sup>5</sup> Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, **24**, 35 (1939)

# DIPHOSPHOPYRIDINE NUCLEOTIDE AS AN ESSENTIAL FACTOR IN THE METABOLISM OF TESTOSTERONE BY THE LIVER\*

Sirs

The enzymic destruction of testosterone by the liver tissue of various species of animals has been previously reported from this laboratory<sup>1</sup> It was shown that oxidation is directly or indirectly involved in this process Chicken liver converts testosterone largely to 17-ketosteroids These are not ordinarily found as products of testosterone metabolism in rat liver<sup>2</sup> Since rat liver will destroy 17-ketosteroids formed by chicken liver, it has been postulated that the rat liver contains both the enzyme systems found in chicken liver and an additional system which destroys the 17-ketosteroids

Substrate	Concentration	Rate in presence	Rate with buffer
		of substrate	only
	$M$	$\gamma$ per gm per hr	$\gamma$ per gm per hr
Succinate	0.05	107	105
Pyruvate	0.05	119	126
Adenosine-5-phosphate	0.001	126	125
Citrate	0.001	269	111
DPN	0.001	209	111
Nicotinamide	0.04	228	125

Using the methods previously described,<sup>2,3</sup> we have studied the effect of various hydrogen carriers and substrates on the rate of destruction of testosterone by minced liver tissue Thus far the only active compounds have been diphosphopyridine nucleotide and citrate The effect of these compounds on the destruction of testosterone is shown in the table

The importance of the role of DPN in the destruction of testosterone is also shown by the fact that the addition of nicotinamide to the incubation medium as an inhibitor of diphosphopyridine nucleotidase also considerably increases the destruction of testosterone by normal rat liver tissue The effects are not due to the general acceleration in tissue metabolism,

\* This investigation was aided by grants from Ciba Pharmaceutical Products, Inc., Summit, New Jersey, The International Research Division of the Donner Foundation, Philadelphia, Pennsylvania, and the Medical Research Fund of the University of Utah

<sup>1</sup> Samuels, L. T., and Pottner, M., *Federation Proc.*, **6**, 287 (1947)

<sup>2</sup> Samuels, L. T., McCauley, C., and Sellers, D. M., *J. Biol. Chem.*, **168**, 477 (1947)

<sup>3</sup> Samuels, L. T., *J. Biol. Chem.*, **168**, 471 (1947)

Since a number of the other substances added increase the oxidative respiration of the tissue as much or more than DPN

Since a large portion of the additional testosterone destroyed when DPN is added appears as 17-ketosteroids, it would seem that DPN acts differentially on the first portion of the postulated two-step system. The intermediate 17-ketosteroids are produced so rapidly that a significant concentration accumulates. The increased concentration of the intermediate then increases the rate of its destruction in the second phase of the reaction.

The discovery of diphosphopyridine nucleotide as an important factor in the destruction of testosterone by rat liver tissue not only indicates the specific enzymic nature of the reaction, but gives additional support to the hypothesis of the stepwise breakdown involving the intermediate formation of 17-ketosteroids.

Citrate speeds up the destruction of testosterone without production of 17-ketosteroids. It, therefore, appears to act through a different enzyme system. Here again the effect appears specific, and it is not due to depression of calcium ion since addition of the latter does not affect the rate of reaction.

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# ON THE INTERACTION OF AVIDIN AND OLEIC ACID

Sirs

Under appropriate conditions, oleic acid replaces biotin for several lactic acid bacteria <sup>1 2</sup> This growth-promoting action of oleic acid is not nullified by excess avidin for *Streptococcus faecalis* R<sup>2</sup> or for several other organisms (Table I) Egg white did, however, block utilization of oleic acid by *Lactobacillus arabinosus* (Table I) This result is obtained whether oleic acid is added alone, as Tween 80, or as the free acid with Tween 40 Purified samples of ovalbumin, conalbumin, and lysozyme were ineffective in preventing utilization of oleic acid Purified avidin (3500 units per gm) was

TABLE I

Addition to 10 cc. of biotin deficient medium	Galvanometer readings*							
	<i>L. arabinosus</i>		<i>L. casei</i>		<i>L. delbrueckii</i>		<i>L. mesenteroides</i>	
		0.5 cc. di luted egg white per 10 cc.†		0.5 cc. di luted egg white per 10 cc.		0.5 cc. di luted egg white per 10 cc.		0.5 cc. di luted egg white per 10 cc.
None	90	100	80	98	95	98	75	97
Biotin, 10 mγ	28	100	35	99	46	95	53	97
Tween 80, 10 mg	37	100	21	22	52	45	55	48

The basal medium and culture technique were those described previously <sup>2</sup>

\* Per cent of incident light transmitted The uninoculated medium was set at 100 per cent light transmission 20 hour readings for *L. arabinosus* and *L. delbrueckii*, other readings at 48 hours

† Prepared by adding 10 cc of fresh egg white to 90 cc of sterile, 0.9 per cent sodium chloride solution

effective Furthermore, the oleic acid "inactivating" potencies of raw egg white and of avidin were directly proportional to the biotin-binding activities of the two preparations 0.1 γ of the purified avidin, sufficient to combine with only 0.2 mγ of biotin, nullified the growth-promoting action of 50 γ of oleic acid Thus interaction of avidin with oleic acid cannot be stoichiometric, as is that with biotin <sup>3</sup> The possibility that growth-promoting effects of oleic acid for this organism may depend upon traces of biotin present as an impurity appears ruled out by several independent lines of evidence For example, growth promotion by oleic acid is not prevented

<sup>1</sup> Williams, V. R., and Fieger, E. A., *J. Biol. Chem.*, 166, 335 (1946)

<sup>2</sup> Williams, W. L., Broquist, H. P., and Snell, E. E., *J. Biol. Chem.*, 170, 619 (1947)

<sup>3</sup> Eakin, R. E., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, 140, 535 (1941)

by concentrations of the biotin analogue,  $\gamma$ -(3,4-ureylenecyclohexyl)-butyric acid, sufficient to prevent the action of biotin (Table II).

TABLE II

$\gamma$ (3,4 Ureylenecyclohexyl) butyric acid, $\gamma$ per 10 cc	Oleic acid		Biotin	
	$\gamma$ per 10 cc	Galvanometer readings*	m $\gamma$ per 10 cc	Galvanometer readings*
0	0	90	0	90
0	50	66	1	58
300	50	70	1	70
1000	50	67	1	94

\* 24 hour readings, test organism, *L. arabinosus*

The close physiological relationship between oleic acid and biotin indicated by their common effect upon growth is further emphasized by the ability of avidin to nullify the effect of each compound. The mechanism of its action, in this instance, is unknown.

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# THE EFFECT OF GROWTH ON THE INCORPORATION OF GLYCINE LABELED WITH RADIOACTIVE CARBON INTO THE PROTEIN OF LIVER HOMOGENATES\*

Sirs

It has been established in recent years that the proteins of the body are in a continuous state of flux, constantly undergoing both synthesis and breakdown<sup>1</sup> It follows from this concept that growth can result either

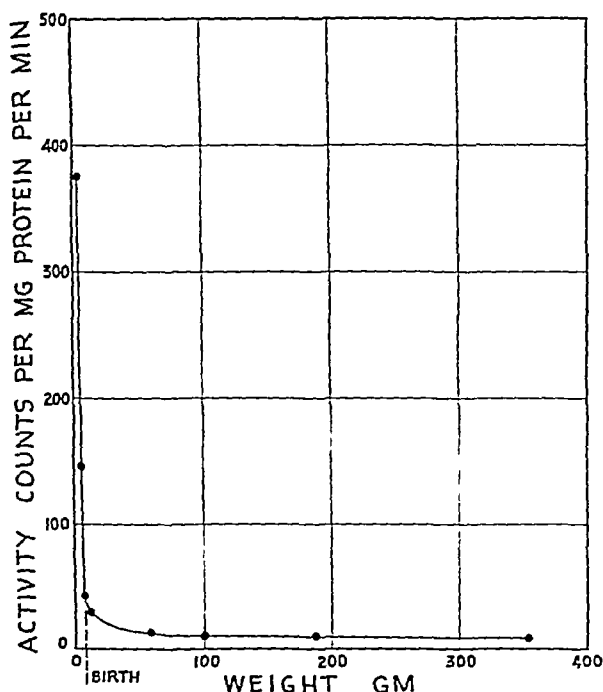


FIG 1 Uptake of C<sup>14</sup>-methylene-labeled glycine by proteins of homogenates of rat liver as a function of body weight The highest point on the left side of the graph represents whole embryo homogenate

from an acceleration of protein synthesis or an inhibition of protein decomposition Rittenberg and Shemin, from unpublished experiments on the rate of protein formation in regenerating liver, concluded that, "Growth is the result of an inhibition rather than the initiation or acceleration of reac-

\* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the National Cancer Institute, United States Public Health Service

<sup>1</sup> Schoenheimer, R, The dynamic state of body constituents, Cambridge (1942)

tions"<sup>2</sup> Our interpretation of experimental data to be reported below is entirely to the contrary, namely, *growth of tissues is the result of increased activity of protein synthesis*

The effect of growth was studied by determining the uptake of  $C^{14}$  from methylene-labeled glycine by the proteins of liver homogenates. Animals at various stages of development were used, 250 mg of liver were removed and homogenized in a synthetic medium.<sup>3</sup> Details of the experimental procedures will be published later. The protein was collected on filter paper, dried at 100°, weighed, and its radioactivity determined. The results are expressed in Fig. 1, from which it can be seen that the rate of incorporation of the label into the protein is strikingly high in the fetus, as contrasted with the adult animal. The rate of synthetic activity decreases rapidly in early life and more slowly as maturity is approached.

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<sup>2</sup> Rittenberg, D., and Shemin, D., in Green, D. E., *Currents in biochemical research*, New York, 272 (1946).

<sup>3</sup> Friedberg, F., Winnick, T., and Greenberg, D. M., *J. Biol. Chem.*, **171**, 441 (1947).

<sup>4</sup> United States Public Health Service Senior Research Fellow, 1947-48.

# INFRA-RED ABSORPTION SPECTRA OF TOCOPHEROLS AND RELATED STRUCTURES\*

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The present report is based on an investigation of the infra-red absorption spectra of natural and synthetic vitamin E substances and structurally related compounds. Such spectra can be helpful through rapid identification of chemical products in the isolation and synthesis of tocopherols. It is also hoped that they may aid in the identification of the various tocopherols and oxidative products which might be isolated from the blood and urine of patients fed vitamin E extracts. This can be important, as it has recently been demonstrated that  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherols show quantitative differences in physiological activity in patients suffering from progressive muscular dystrophy.<sup>1</sup>

## Methods

The infra-red spectra were obtained on the Hardy two beam spectrophotometer (1) and were recorded in the range from 2 to 12  $\mu$ . The instrument has been reported in detail by Furchgott *et al* (2), but since then it has been designed to record automatically with a General Motors breaker type amplifier and a Brown potentiometer recorder. A cam control for the slits was also introduced in order to obtain a uniform intensity level over the region observed.

The samples were investigated as solid films on rock salt plates. Most of them were oils, smears being made between two NaCl plates, others were prepared from pyridine and carbon tetrachloride solution, the solvent being evaporated on a hot-plate. The oil smears gave better resolution and definition, however, there was a small shift in some bands when a crystalline film was used.

## EXPERIMENTAL

The absorption spectra shown here are copies of original tracings from the automatic recorder, preserving band position and intensity but adjusting the base lines. The adjustment of base lines was necessitated

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<sup>1</sup> Milhorat, A. T., personal communication



because the cam control of the slit widths was not theoretically correct in giving a uniform intensity distribution. However, this did not affect the position of the absorption bands. The absorption curves of the following substances were obtained: natural  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocopherol, natural  $\alpha$ -tocopherol acetate, succinate, and palmitate, natural  $\gamma$ -tocopherol palmitate, synthetic  $\alpha$ -tocopherol and its acetate,  $\alpha$ -tocopherylquinone,  $\alpha$ -tocopherylhydroquinone triacetate, 2,5,6-trimethylhydroquinone, 1,4-naphthoquinone, 2-methyl-1,4-naphthoquinone, and 1,4-benzoquinone.<sup>2</sup>

The infra-red absorption spectra of synthetic *dl*- $\alpha$ -tocopherol and its acetate, although not shown in this paper, were found to be nearly identical with the spectra of natural *d*- $\alpha$ -tocopherol and its acetate respectively. The isolation of  $\delta$ -tocopherol was reported by Weisler, Robeson, and

TABLE I  
*Absorption Maxima in Hydroxyl Region*

Compound	Type O—H	Wave-length
		$\mu$
$\alpha$ -Tocopherol	Phenolic	3.02
$\gamma$ -Tocopherol	"	3.02
$\delta$ -Tocopherol	"	3.01
2,5,6-Trimethylhydroquinone	"	3.03
$\alpha$ -Tocopherylquinone	Alcoholic	2.93
$\alpha$ -Tocopherol succinate	Acid hydroxyl	3.?

Baxter<sup>3</sup> and we were fortunate in obtaining a small sample from them (3). There is a fourth vitamin E substance known as  $\beta$ -tocopherol which we have not studied at the present.

### *Analysis of Spectra*

The infra-red absorption bands which can be assigned to specific atomic linkages will be discussed first.

*O—H Absorption*—Many of the compounds studied have one or more hydroxyl groups, whose linear vibrations appear in the 3  $\mu$  region of their spectra. Table I lists the wave-lengths of the absorption maxima observed in this region. It can be seen that the phenolic hydroxyl groups absorbed between 3.01 and 3.03  $\mu$ , while the alcoholic hydroxyl group absorbed about 2.93  $\mu$ . These values appear to be higher than the 2.75 to 2.80  $\mu$  absorption range for unbonded O—H groups. Furchgott *et al.* (4) have

<sup>2</sup> We wish to express our gratitude to Dr. Ade T. Milhorat of The New York Hospital, for obtaining many of these compounds from Distillation Products, Inc. for us.

<sup>3</sup> Meeting of the American Chemical Society, Chicago, September, 1946.

postulated intermolecular bonding for this shift to longer wave-lengths in estrogens, which also have a phenolic structure. The acid hydroxyl of  $\alpha$ -tocopherol succinate gave a side bump in the alcoholic hydroxyl region. However, the major absorption from this acid hydroxyl was probably shifted into the C—H region because of the strong hydrogen bonding associated with acid groups (5).

**C—H Absorption**—Other workers (6) have demonstrated that C—H groups of benzene rings absorb near  $3.25 \mu$ , while bands due to aliphatic type C—H linkages occur near  $3.41 \mu$ . In all these compounds studied here which have the long phytyl chain, the absorption maxima for the

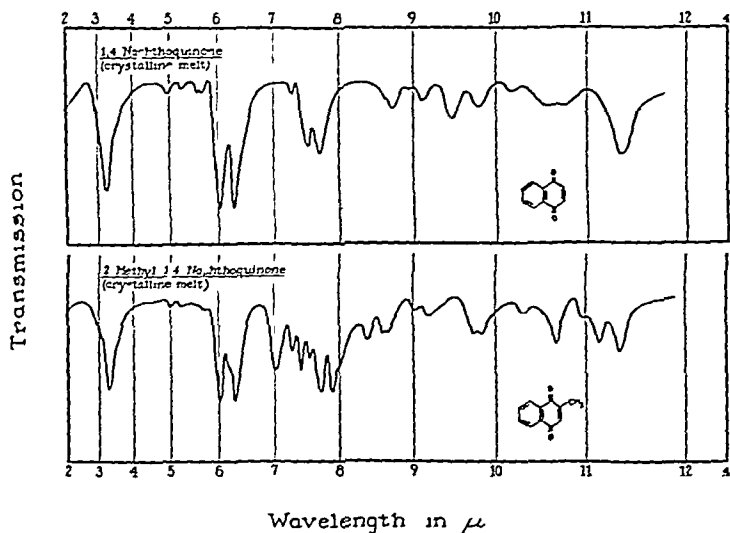


FIG 1

linear C—H linkage occurred at about  $3.41 \mu$ . In the case of the quinones which contain all or mainly aromatic C—H linkages, the absorption band occurred nearer  $3.23 \mu$ .

Absorption bands also arise in the region near  $7 \mu$  from the angular vibration of C—H linkages (6). A series of bands is resolved between  $6.7$  and  $7.15 \mu$  which can be attributed to the aliphatic type C—H linkages (6). A clear cut example of this is shown through comparison of naphthoquinone and 2-methylnaphthoquinone (Fig 1). In the latter compound, a band appeared at  $7.02 \mu$  which did not occur in the former. This is probably due to angular vibrations of hydrogen atoms of the methyl group attached to the aromatic nucleus. Such vibrations, in the case of methyl groups in saturated aliphatic molecules, appear to absorb at a somewhat lower wave-length (see the spectra presented by Barnes *et al* (6)).

**CH<sub>3</sub> Absorption**—In the spectra of tocopherols, a band consistently occurred near 7.27  $\mu$ . On the basis of other studies (6) on the absorption of the CH<sub>3</sub> groups and our comparison between methylnaphthoquinone and non-methylated quinones, this band was attributed to the methyl groups present in these vitamin E compounds.

Naphthoquinone showed a band at 7.28  $\mu$ , but this is very weak compared with the bands near this wave-length found in the spectra of the tocopherols and of methylnaphthoquinone (Fig. 1).

**C=O Absorption**—The three types of carbonyl groups encountered in this study were ester, acid, and quinone. The ester type, as found in

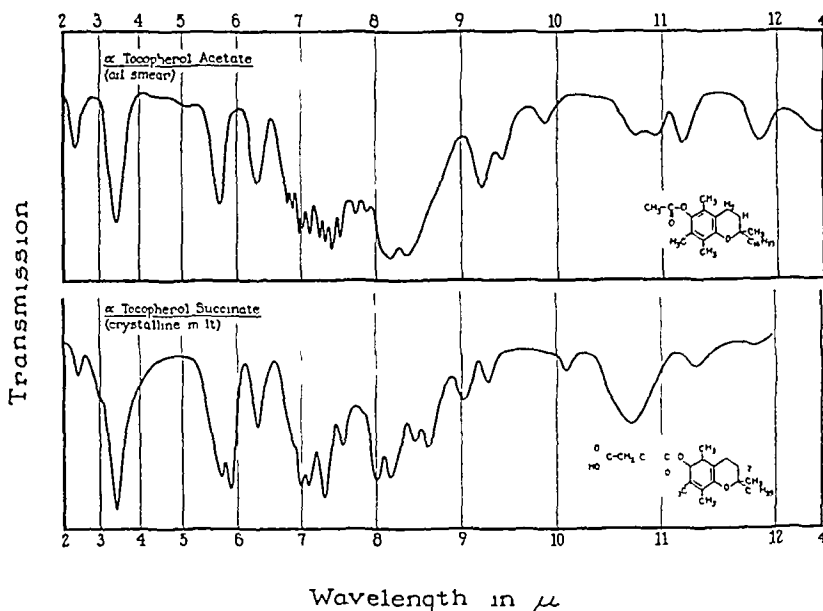


FIG. 2

$\alpha$ -tocopherol acetate, succinate, palmitate, and  $\gamma$ -tocopherol palmitate absorbed at about 5.70  $\mu$  (Fig. 2). In the  $\alpha$ -tocopherol succinate we also find an acid type carbonyl which absorbed at 5.87  $\mu$ .

The naphthoquinones and 1,4-benzoquinone were examined to help locate the absorption band arising from the quinone type carbonyl in  $\alpha$ -tocopherylquinone. It can be seen from Fig. 3 that all the quinones gave a strong band at 6.03  $\mu$ . This band has been assigned to the quinone type C=O vibration.

**C=C Absorption**—The conjugated C=C system of the benzene and substituted benzene molecules has been previously shown (6) to give rise to a strong band in the 6.15 to 6.35  $\mu$  region. In the spectra of phenol (6) a strong band appears at about 6.27  $\mu$ . A strong band occurred consistently near 6.3  $\mu$  in these tocopherols and has been assigned to the phenolic type

structure Such a band also appeared in the quinones, but was more intense This may be due to the more highly conjugated systems of the quinones

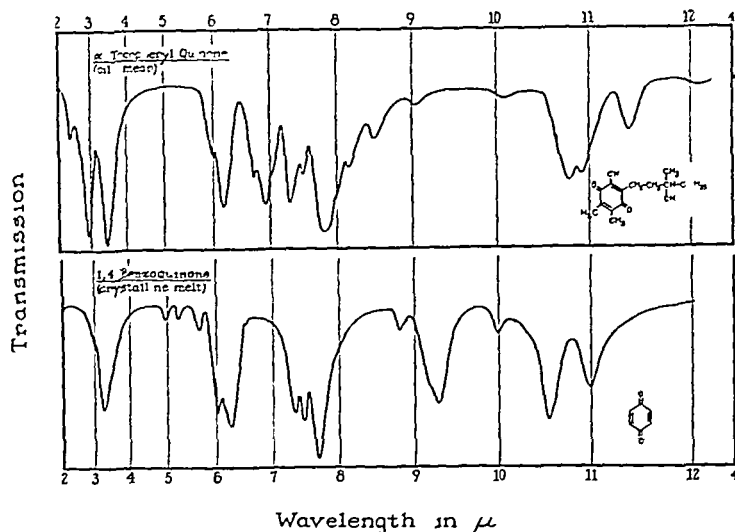


FIG 3

TABLE II

Probable Absorption Maxima of Phenolic C—O Linkage

Compound	Wave length
	$\mu$
$\alpha$ -Tocopherol	7.85
$\gamma$ -Tocopherol	8.17
$\delta$ -Tocopherol	8.28
$\alpha$ -Tocopherol acetate	8.18
$\alpha$ -Tocopherol palmitate	8.06
$\alpha$ -Tocopherol succinate	8.16
$\gamma$ -Tocopherol palmitate	8.14
$\alpha$ -Tocopherylhydroquinone triacetate	8.08
2,5,6-Trimethylhydroquinone	8.0*

\* See the text

**Phenolic C—O Absorption**—It appears probable that the intense absorption band near 8  $\mu$  in the tocopherols results from the C—O vibration of the phenolic hydroxyl. Previous work (4, 6) has indicated that C—O linkages, where the C has one double bond, give rise to bands in this region. The influence of methylation and esterification was observed on the 8  $\mu$  band.

Substitution of methyl groups on the tocopherol molecule resulted in a shift of the  $8\ \mu$  band to a shorter wave-length. Table II shows this shift as one goes from the monomethyl- $\delta$ -tocopherol to the trimethyl- $\alpha$ -tocopherol. This band occurred at  $8.28\ \mu$  in  $\delta$ -tocopherol,  $8.17\ \mu$  in  $\gamma$ -tocopherol, and at  $7.85\ \mu$  in  $\alpha$ -tocopherol. Esterification of  $\alpha$ -tocopherol appeared to shift the  $8\ \mu$  band to a longer wave-length (Table II). The  $7.85\ \mu$  band of  $\alpha$ -tocopherol was shifted to  $8.18\ \mu$  in the acetate,  $8.06\ \mu$  in palmitate, and  $8.16\ \mu$  in the succinate.

The trimethylhydroquinone which also has the phenolic type C—O linkage gave a split band near  $7.6\ \mu$  and another band at  $8.23\ \mu$ . It is

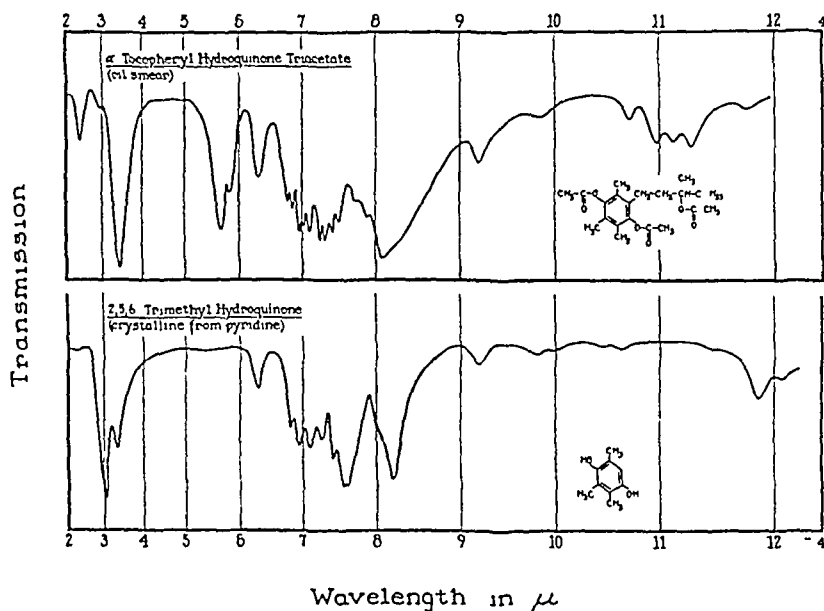


FIG 4

not known at this time which of these bands resulted from the phenolic hydroxyl C—O vibration (Fig 4).

*Other Absorption Bands*—In the spectra of this group of tocopherols, there are other absorption bands which cannot as yet be assigned to specific interatomic vibrations. Many of these “unassigned” bands occurred between 10 and  $12\ \mu$ . Absorption bands occurring near  $10\ \mu$  probably arise from C—C vibrations (6). Many of the others undoubtedly result from complex vibrations involving the chromanol nucleus itself as well as the long phytol chain. With this possibility in view, cross-comparisons were made of all the absorption spectra obtained in this study to see whether any relationship existed.

The spectra of the tocopherols studied here showed a band near  $8.6\ \mu$

This band was probably masked in  $\alpha$ -tocopherol acetate by the broad, intense bands near this region. However, its medium intensity and its consistency indicated that it might be a characteristic band of the tocopherol molecule. In this respect, it is interesting to observe the marked increase in intensity of this  $8.6 \mu$  band in the  $\alpha$ - and  $\gamma$ -tocopherol palmitates (Fig 5). This increase in intensity is probably due to an effect by the long fatty acid side chain. Examination of spectra published by Barnes *et al* (6) of esters of long chain fatty acids demonstrated the presence of a strong band near this wave-length.

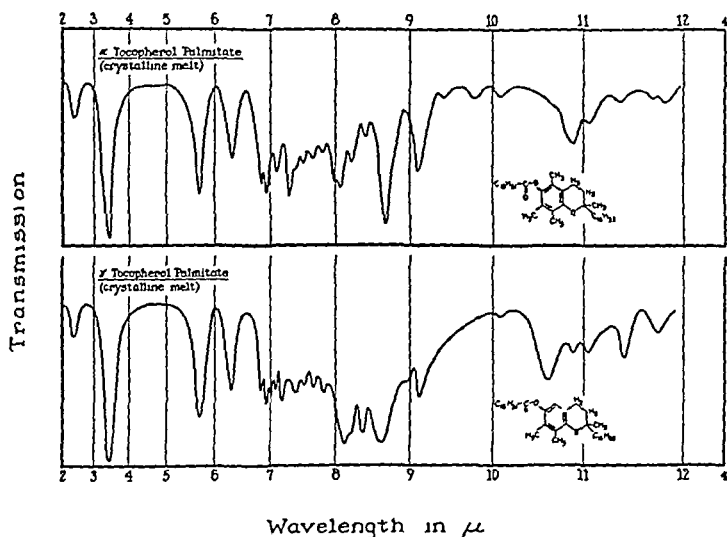


FIG 5

The region between  $10.6$  and  $11.0 \mu$  in the spectra of the tocopherols and their derivatives contained one or two strong bands. The variations of wave-lengths of these bands in the free tocopherols make them useful for differentiation. They are probably characteristic bands of the tocopherol structure.

There seemed to be some consistency among the compounds as to absorption bands which occurred near  $11.4$  and  $11.8 \mu$ . However, the large variation in intensity of these bands offered too much difficulty in interpretation.

An absorption band occurred in the tocopherols and the two oxidative products of  $\alpha$ -tocopherol near  $2.4 \mu$ . This wave-length did not correspond to the atmospheric water vapor bands in this region. Several estrogens which contain a phenolic configuration in Ring A were studied, and no

bands near  $2.4 \mu$  were found. We are not certain whether this band is an artifact or an overtone.

In conclusion, it may be pointed out that the various tocopherols, as well as their derivatives, may be easily distinguished by their infra-red absorption spectra (Fig 6). The tocopherol structure itself appears to be characterized by certain absorption bands. These bands include several assigned absorption bands resulting from vibrations of the phenolic

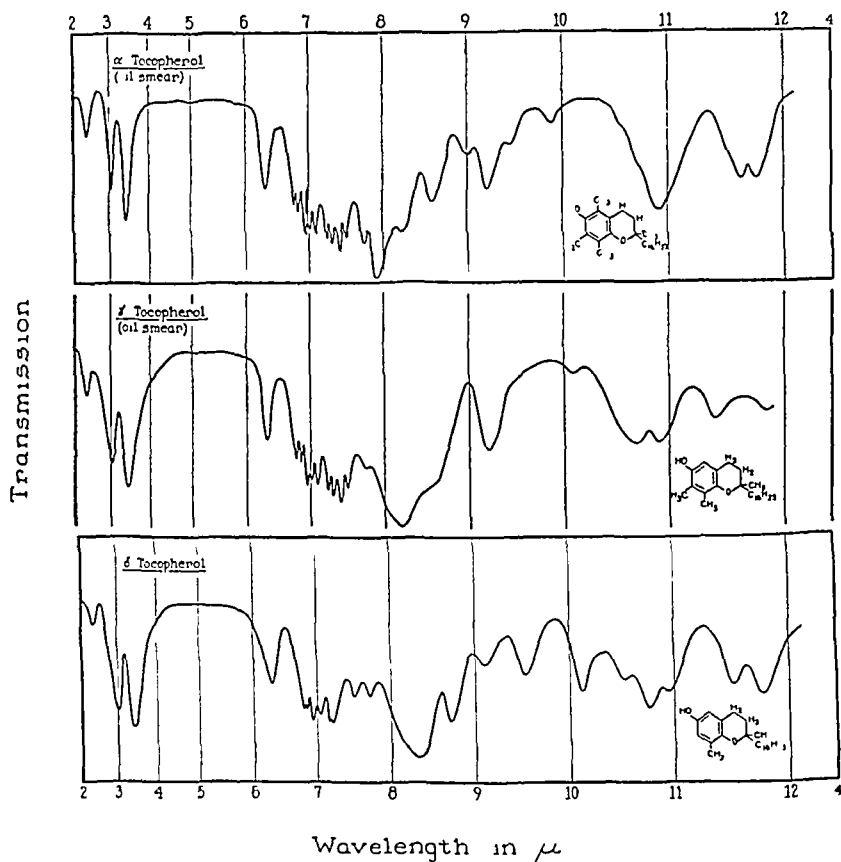


FIG 6

hydroxyl, the benzene ring, and the phenolic C—O linkage, as well as certain "unassigned" absorption bands occurring near  $8.6$  and  $10.9 \mu$ .

#### SUMMARY

- 1 The infra-red absorption spectra, from  $2$  to  $12 \mu$ , of nine free and substituted tocopherols and six related structures have been presented.
- 2 Certain absorption bands in the spectra have been discussed in relation to the chemical structure of tocopherols.
- 3 Assigned absorption bands near  $3.0$ ,  $6.3$ , and  $8.0 \mu$  in addition to

unassigned bands near 8.6 and 10.9  $\mu$  are characteristic of the tocopherol structure

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# APPLICATION OF AN IMPROVED GLUCURONIDASE ASSAY METHOD TO THE STUDY OF HUMAN BLOOD $\beta$ -GLUCURONIDASE\*

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Previous conditions in which phenolphthalein glucuronide was employed as substrate (1) have proved unsatisfactory for the determination of glucuronidase in plasma, serum, and laked blood cells. Thus, low glucuronidase activities such as frequently occur in plasma could not be accurately measured or even approximated, owing to a combination of circumstances, *i.e.*, too short an incubation period often coupled with interference by a turbid state of the plasma and the presence in it of biliary and carotenoid pigments. Moreover, solutions of laked erythrocytes could not be assayed because of the red color of the hemoglobin. These difficulties have now been largely overcome by the introduction of a deproteinizing procedure and by otherwise modifying the conditions of assay. It has been possible also to simplify greatly the biosynthetic process of manufacturing the substrate, phenolphthalein glucuronide, which is now in demand.<sup>1</sup>

The interpretation of blood glucuronidase values is handicapped by our lack of knowledge concerning the factors which control the level of this enzyme in the blood, *e.g.*, we do not know to what extent the plasma glucuronidase is influenced by hormonal factors. The nature and significance of the distribution of  $\beta$ -glucuronidase between the plasma and the formed elements of the blood is likewise not understood. As a result of observations made on blood glucuronidase with the improved assay technique, some of the desired information has been obtained. An account of the method and its use in these experiments is given in the present paper.

## Method

*Simplified Preparation of Substrate, Phenolphthalein Glucuronide*—The filtered, toluene-free urine obtained from rabbits receiving injections of sodium phenolphthalein phosphate<sup>2</sup> was acidified to Congo red paper with

\* Aided by a grant from the Otho S. A. Sprague Memorial Institute.

<sup>1</sup> Private communications.

<sup>2</sup> This product is now made more conveniently than before (2). First, dry chloroform (40 cc.) and then a mixture of (redistilled) phosphorus oxychloride (50 cc.) and chloroform (50 cc.) was added with mechanical stirring to 50 gm. of phenolphthalein in

6 N hydrochloric acid with vigorous shaking, and 800 cc portions of urine were extracted with four successive 125 cc portions of ethyl acetate. The ethyl acetate phase was centrifuged, dried by decantation through cotton, and reduced to small volume *in vacuo* at 50°. This was added to an excess of saturated cinchonidine in ethyl acetate. The cinchonidine derivative of phenolphthalein glucuronide so obtained was crystallized by dissolving it in the minimum quantity of hot methyl alcohol and adding 4 volumes of hot ethyl acetate. One more such crystallization was required in order to obtain a product which, according to analyses for hydrolyzable phenolphthalein, is pure phenolphthalein mono- $\beta$ -glucuronide with 1 molecule of methyl alcohol of crystallization. The yield of product has been greatly improved by this process.

*Method for Blood Glucuronidase*—Plasma and formed elements of the oxalated or heparinized blood (14 cc divided between two Wassermann tubes) were separated by centrifugation. The buffy coat was aspirated with a capillary pipette, after removal of the plasma, and was laked with 10 cc of distilled water after alternate freezing (in a carbon dioxide snow-acetone mixture) and thawing at room temperature, to facilitate lysis. The red cells were laked with three volumes of distilled water and the total volume was recorded. The plasma was usually filtered through a Seitz filter before sampling it for assay. Blood serum was sampled without any preliminary treatment.

Into two Wassermann tubes were pipetted 0.1 cc of glucuronidase-containing solution, 0.8 cc of 0.1 M acetate buffer (1), pH 4.5, and 0.1 cc of 0.01 M phenolphthalein glucuronide (1). A third Wassermann tube containing the enzyme and buffer but not the substrate served as the control. The digests were then incubated at 38° and the time recorded. For blood plasma, serum, and cells, an incubation period of between 15 and 24 hours was required in contrast to a 1 to 5 hour period for most tissues.

a 1 liter round bottom flask cooled in an ice bath. To this was added dropwise dry pyridine (40 cc), with stirring continued for a total of 3 to 5 hours. The next day, 150 cc of distilled water were added in small quantities to the ice-cooled reaction flask, followed by an excess of 40 per cent sodium hydroxide (approximately 300 cc). Upon cooling, needle-like crystals (sodium phosphate) appeared and were removed by filtration. When an excess of concentrated hydrochloric acid (Congo red paper) was added to the aqueous phase, phenolphthalein diphosphoric acid precipitated as a gum which could be conveniently separated from the mixture with a glass stirring rod. This gum was warmed in a porcelain dish on a boiling water bath and 20 cc of concentrated sodium hydroxide (100 gm plus 100 cc of distilled water) were added gradually with stirring. Solution of the gum was completed with distilled water (final volume 200 cc). 3 cc of this phenolphthalein phosphate were brought to neutrality with weak alkali and diluted to 10 cc with distilled water. This amount was injected subcutaneously daily at two widely separated sites on the skin of each rabbit for 6 days. The animals received carrots and cabbage, water being withheld. The urine collections under toluene were completed on the morning of the 8th day.

At the end of the incubation period, 10 cc of 5 per cent trichloroacetic acid was added to each tube with thorough mixing. To digests prepared from laked cells was added 10 cc of 10 per cent trichloroacetic acid. This step served to stop the reaction and to deproteinize the digest. The time was recorded. The tubes were centrifuged at high speed for 10 minutes. The contents were decanted through cotton plugs held in the stem of small glass funnels into colorimeter tubes marked at 6 cc. The colorimeter tubes contained 2.5 cc of an alkaline reagent mixture (200 cc of glycine buffer of pH 10.45 (1) plus (a) 50 cc of 0.5 N sodium hydroxide in the case of digests deproteinized with 5 per cent trichloroacetic acid or (b) 50 cc of 1.0 N alkali when 10 per cent trichloroacetic acid was used). The precipitate remaining after decanting was suspended in 10 cc of distilled water, centrifuged, and the supernatant decanted through the same filter. Washing of the tube and funnel was continued until the filtrate amounted to 6 cc. 0.1 cc of 0.01 M phenolphthalein glucuronide was added to the control tube, which was used in the colorimeter to obtain the setting at 100. After thorough mixing, readings were made with the 6.0 cc well and the 540 m $\mu$  filter. The *L* (optical density) values were substituted in a phenolphthalein calibration curve (1) previously prepared with the present volumes of the various constituents of the enzyme digest.

When fresh reagents are employed, it is important to check the pH of the final alkalinized mixture (pH 10.2 to 10.4) or the color development of a known amount of phenolphthalein.

#### Calculations

##### *Serum, Plasma, or Body Fluids*

$$\begin{aligned} \text{Micrograms phenolphthalein liberated in digest} &\times \frac{1}{\text{hrs of incubation}} \\ &\times \frac{100}{\text{cc fluid analyzed}} = \text{units glucuronidase per 100 cc fluid} \end{aligned}$$

##### *Laked Blood Cells<sup>3</sup>*

$$\begin{aligned} \text{Micrograms phenolphthalein liberated in digest} &\times \frac{1}{\text{hrs of incubation}} \\ &\times \frac{\text{volume laked cells}}{0.1} \times \frac{100}{\text{volume original blood specimen}} \\ &= \text{units glucuronidase per 100 cc whole blood} \end{aligned}$$

<sup>3</sup> It has been considered best for the present to relate the activity of laked blood cells to a 100 cc volume of whole blood. The buffy coat, especially, consists of a heterogeneous mixture of cell types and so the use of units based on the number of cells only would be meaningless in the opinion of a histologist. However, it should be pointed out that the values for buffy coat glucuronidase do not properly indicate the great magnitude of the glucuronidase activity which must exist in the white cells as compared to an equivalent number of tissue cells.

*Tissues*

Micrograms phenolphthalein liberated in digest  $\times \frac{1}{\text{hrs of incubation}}$

$$\times \frac{\text{volume extract}}{0.1} \times \frac{1}{\text{gm tissue}} = \text{units glucuronidase per gm tissue}$$

The changes which have been introduced in the method have in no way altered the principle upon which the test was based originally, *i e*, the

TABLE I  
*Distribution of  $\beta$ -Glucuronidase in Blood*

Healthy adults	Glucuronidase activity			Morphology of white blood cells			
	Plasma, units per 100 cc	Red cells, units per 100 cc whole blood	Buffy coat, units per 100 cc whole blood	No per c.mm	Polymor- phonuclear leucocytes	Lym- phocytes	Other cells
					<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
C ♂	37	0	818	7,800	27	73	0
S ♂	84	5	378	5,500	61	37	2
G ♂	140	0	307	5,400	33	60	7
P ♂	121	0	614	9,500	37	62	1
M ♂	0	10	780	21,400	68	30	2
L ♂	37	0	384	8,500	73	27	0
H ♂	0	0	353	12,700	58	42	0
J ♂	150	0	261	8,200	66	30	4
L ♂	181	18	425	9,500	60	40	0
II ♂	79	7	655	10,200	67	33	0
S ♂	118	3	647	7,400	86	14	0
G ♀	96	0	318	4,800			
B ♀	84	0	426	7,900	47	47	6
S ♀	179	0	528	7,000	57	40	3
W ♀	230	0	423	13,500	90	10	0
G ♀	86	8	342	8,200	45	50	5

colorimetric determination of phenolphthalein liberated by enzymatic hydrolysis of phenolphthalein glucuronide. Optimal conditions of hydrolysis have been maintained and very good agreement in duplicate analyses was obtained as before (1). The present procedure has greater flexibility in its application and is not affected by the physical state or the pigment content of the specimen. Great economy of substrate is achieved without sacrificing any of the desirable features of the original method. A few data on the blood and tissues of cancer patients have already appeared (3).

## EXPERIMENTAL

The distribution of glucuronidase activity between plasma, erythrocytes, and the buffy coat was determined in sixteen subjects with the method

described. These values are compared with white cell morphology in Table I.

Platelet-rich plasma was prepared by slow centrifugation of 14 cc of heparinized blood. After removal of this platelet-rich plasma, the blood

TABLE II  
*Study of Platelet Glucuronidase Activity*

Subject	No. of platelets in plasma per c.mm	Platelet rich plasma, units per 100 cc	Platelet free plasma, units per 100 cc	Platelet poor buffy coat, units per 100 cc whole blood
S	140,000	166	103	759
Y	170,000	146	118	854
B	300,000	76	73	580
L	440,000	58	37	874
S	400,000	164	171	200
W	500,000	59	72	333

TABLE III  
*Effect of Repeated Washing with Tyrode's Solution on Buffy Coat Glucuronidase*

	Subject 1*		Subject 2*	
	Activity, units per 100 cc. blood	Per cent total buffy coat glucuronidase	Activity, units per 100 cc. blood	Per cent total buffy coat glucuronidase
1st washing	125	7.3	111	8.9
2nd "	51	3.0	104	8.3
3rd "	28	1.6	60	4.8
4th "	68	4.0	98	7.8
5th "	14	0.8	21	1.7
6th "	44	2.6	71	5.6
7th "	64	3.8	29	2.3
Water-laked washed buffy coat	200	11.8	292	23.2
Freezing-laked residue after water-laking	1110	65.1	472	37.4
Total buffy coat glucuronidase	1704		1258	

\* White cell count of Subject 1, 8400 per c mm, of Subject 2, 10,800

cells were centrifuged at high speed and the platelet-poor buffy coat aspirated and laked with 10 cc of water after an intermittent freezing-thawing procedure. Glucuronidase determinations were done on the plasma before and after Seitz filtration and on the laked buffy coat. These values have been correlated with plasma platelet counts in Table II.

In Table III, two cases are illustrated of the effect of repeated washing with Tyrode's solution on buffy coat glucuronidase. The buffy coat of

14 cc of blood was separated and evenly suspended with gentle stirring in 10 cc of Tyrode's solution. The mixture was centrifuged and the supernatant removed for glucuronidase assay (first washing). After seven such washings, the buffy coat was laked with distilled water (10 cc), centrifuged, and the supernatant (water-laked buffy coat) assayed. The residue was subjected to alternate freezing and thawing at room temperature. The material was suspended in 10 cc of  $H_2O$ , centrifuged, and the supernatant assayed (freezing-laked residue). The white cells remained intact during washing with Tyrode's solution. However, suspending them in distilled water afterwards accomplished almost complete laking.

### *Results*

From Table I it is clear that the red cells contain little or no glucuronidase and that the buffy coat contains the major portion of the blood glucuronidase activity. Similar findings have been made with the blood of the mouse, rat, rabbit, and dog. There does not seem to be any correlation of the buffy coat glucuronidase with the percentage of polymorphonuclear leucocytes and lymphocytes in the blood. Although greater glucuronidase activities are frequently found in the presence of high white cell counts, a strict dependence of the glucuronidase level upon the number of white blood cells is not evident. This may be due in part to variations in the amount of buffy coat which can be recovered for assay.

The platelet-rich plasma contains a small number of white cells which are rich in glucuronidase. The removal of these cells rather than the platelets probably explains the small decrement in activity which follows Seitz filtration (Table II). It may be concluded that the platelets contain little or no glucuronidase activity.

A substantial fraction of the total buffy coat glucuronidase can be removed by repeatedly suspending the cells in Tyrode's solution (Table III). The disruption of the cell membranes by water laking did not release as much enzymic activity as appeared in the lysate after the intermittent freezing-thawing procedure.

### DISCUSSION

The presence of  $\beta$ -glucuronidase activity in blood was first observed and studied by Fishman with a view to demonstrating a possible correlation with the process of the "metabolic conjugation" of the estrogenic hormones. While the blood of castrated female mice showed no change in glucuronidase activity following estrogen injection (4), the blood glucuronidase of women underwent characteristic alterations correlated with the events of pregnancy (3).

It was then clearly established that the greater part of the blood glucuron-

idase resided in the formed elements composed of erythrocytes, platelets, leucocytes, and lymphocytes. The present data strongly indicate that the erythrocytes and platelets may be eliminated as important sources of blood cell glucuronidase. In view of the relatively low glucuronidase activity of normal lymph node tissue (5, 6), one would expect that the lymphocytes should possess less of this enzyme than the leucocytes. On the other hand, the lack of correlation of buffy coat glucuronidase with the relative proportion of lymphocytes and leucocytes would not be in favor of this prediction. For the time being, final conclusions should not be drawn until other experiments are done in which the influence of blood factors affecting glucuronidase<sup>4</sup> have been properly taken into account.

The liberation of  $\beta$ -glucuronidase activity from the buffy coat of the blood by washing with Tyrode's solution is of interest with regard to the question of the distribution of  $\beta$ -glucuronidase in the living cell. In view of the low content of glucuronidase in erythrocytes and in platelets, it seems unlikely that the enzyme is merely adsorbed by the cell membrane from the plasma in a non-specific manner. However, the possibility does exist that the enzyme is a cytoplasmic constituent, capable of being secreted through the cell membrane, since  $\beta$ -glucuronidase does occur in secretions of the glandular epithelium (saliva, gastric juice, urine, spinal fluid, tears, bronchial secretions), the range of glucuronidase activity being 50 to 800 units per 100 cc.

In view of the known secretory functions of malignant neoplastic cells, one may expect to find glucuronidase in both the cells and stroma of cancer tissue.

Under the conditions which obtain *in vivo*, one wonders to what extent plasma or serum glucuronidase may be derived from the white blood cells, either through their breakdown or possibly through secretion. In this regard, it seems likely that the white cells ordinarily contribute little glucuronidase to the plasma, since many normal individuals have practically no plasma glucuronidase activity in the presence of high buffy coat enzymic activity. However, this point should be kept in mind in the study of blood glucuronidase in disease.

The explanation of the function of the enzyme in the white blood cells is not apparent at present. Previously, it has been suggested that  $\beta$ -glucuronidase functions in processes of "metabolic conjugation", with much attention being directed towards the formation of steroid glucuronides. However, another possibility also exists, *e.g.*, other glucuronides of greater molecular weight, such as mucin, hyaluronic acid, chondroitin sulfate, and heparin may be synthesized in part through glucuronidase activity. Such syntheses may or may not be related to estrogen metabolism. In this

<sup>4</sup> Fishman, W. H., Altman, K. I., and Springer, B., *Federation Proc.*, in press.



regard it should be pointed out that intercellular mucin can be increased by the administration of estrogenic hormones (7-11)

#### SUMMARY

The method for determining glucuronidase activity with phenolphthalein glucuronide as substrate has been adapted to the assay of blood glucuronidase. This procedure has the advantages of greater flexibility in its use and of freedom from interfering substances in blood. In addition, the process of manufacturing biosynthetic phenolphthalein glucuronide has been improved and simplified.

By means of this method, it has been possible to show that the major portion of the glucuronidase activity of the blood is concentrated in the leucocytes and lymphocytes of the formed elements, little or none being present in erythrocytes and platelets. A substantial amount of white blood cell glucuronidase can be removed from the intact cells by washing them with Tyrode's solution.  $\beta$ -Glucuronidase activity has been demonstrated in saliva, gastric juice, spinal fluid, urine, and tears, which suggests that the enzyme can be secreted by the glandular epithelium. These observations have been discussed in relation to the possible function of the enzyme in processes of metabolic conjugation.

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# ON THE PROTEOLYTIC ENZYMES OF ANIMAL TISSUES

## VII A PEPTIDASE OF CALF THYMUS\*

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It was noted in an earlier paper of this series (1) that the sera of several animals and the extracts of various tissues (skin, lung, intestinal mucosa, etc.) contain a peptidase which rapidly hydrolyzes L-leucylglycylglycine (L-LGG) to leucine and glycylglycine. This peptidase is different from the previously recognized leucine aminopeptidase, since it does not hydrolyze L-leucinamide (L-LA) and is not activated by manganese ions. Its widespread distribution in tissues has raised the possibility that the tripeptide-splitting enzyme is not derived from the characteristic cells of a particular tissue such as skin or lung, but from cells such as lymphocytes which may be present in that tissue. Furthermore, the occurrence of this peptidase in serum has been attributed to its liberation into the circulating body fluids in the course of the rapid turnover of lymphoid cells (1). This view has received support from the finding that the administration to mice of adrenal cortical extracts or of pituitary adrenotropic hormone results in the elevation of the serum peptidase activity toward L-LGG (2). In the light of the prior demonstration that the rate of turnover of lymphoid tissue is under pituitary-adrenal control (3), this result encourages the conclusion that a significant portion of the serum peptidase activity is derived from the disintegration of lymphoid cells. These developments have prompted the study of the proteolytic activity of extracts of tissues known to be rich with respect to lymphocytes. In the present communication, data are presented concerning some of the proteolytic enzymes found in extracts of calf thymus.

*Proteolytic Activity of Crude Saline Extracts of Calf Thymus*—When calf thymus is extracted with 2 per cent sodium chloride solution, the resulting solution exhibits considerable proteolytic activity toward tripeptides such as L-LGG and diglycylglycine (GGG). As will be noted in Table I, following the addition of manganese ions, the hydrolysis of L-LGG is accelerated and L-LA is split rapidly, thus indicating the presence, in the saline extract, of a manganese-activatable leucine aminopeptidase. The typical substrate for trypsinases, benzoyl-L-argininamide, and a substrate for pepsinases,

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carbobenzoxy-L-glutamyl-L-tyrosine, are not hydrolyzed appreciably, and similar negative results were obtained with carbobenzoxyglycyl-L-phenylalanine, the typical substrate for carboxypeptidases. The presence of a manganese-activatable prolidase (substrate, glycyl-L-proline) could be demonstrated, however. It would appear, therefore, that, in addition to leucine aminopeptidase and prolidase, the saline extract of calf thymus contains appreciable amounts of a tripeptide-splitting peptidase similar to that

TABLE I

*Proteolytic Activity of Saline Extract of Calf Thymus*

Enzyme concentration, 0.28 mg. of protein nitrogen per cc. of test solution

Substrate	pH	Time	Hydrolysis		
			No activator added	0.001 M MnSO <sub>4</sub> present	0.01 M cysteine present
		hrs	per cent	per cent	per cent
L-Leucylglycylglycine	7.8	1	32	72	35
		2	60	103	56
Diglycylglycine	7.8	1	20	12	19
		2	39	25	37
L-Leucinamide	7.7	2	6	57	4
		4	14	94	8
Benzoyl-L-argininamide	5.0	7			3
	7.6	7	2		
Carbobenzoxy-L-glutamyl-L-tyrosine	5.4	6	1		
Carbobenzoxyglycyl-L-phenylalanine	5.1	6			2
	7.6	6	0		
Glycyl-L-proline	8.0	4	5	21	

noted previously in extracts of skin and lung and in serum. As will be shown in this communication, this enzyme hydrolyzes both L-LGG and GGG, and in what follows it will be referred to as lymphopeptidase.<sup>1</sup>

*Partial Purification of Lymphopeptidase*—Appreciable concentration of lymphopeptidase activity may be effected by heating the crude saline extract to 50°, followed by fractional precipitation between 0.4 and 0.7 saturation with respect to ammonium sulfate. Prolonged dialysis against distilled water gives enzyme solutions which are notably enriched in lympho-

<sup>1</sup> Since the lymphoid cells of calf thymus constitute only about 60 per cent of the cellular mass, exception may perhaps be taken to the designation of the tripeptide-splitting enzyme as a constituent of lymphocytes. Support for the use of the term "lymphopeptidase" is provided by unpublished studies on the mesenteric lymph node of the mouse. This tissue, which is characterized by an overwhelming predominance of lymphocytes, also exhibits extremely high proteolytic activity toward L-LGG and GGG.

peptidase and which show little or no leucine aminopeptidase or prolidase activity (Table II). This enzyme preparation has been used in the experiments reported in the succeeding sections of this paper.

As in the case of other tissue extracts (1), such solutions of lymphopeptidase, under our experimental conditions, hydrolyze L-LGG and GGG with the kinetics of a zero order reaction (*cf* Table III), and the rate may therefore be defined by a constant  $K^0$  which equals per cent hydrolysis per minute. In Table III, the proteolytic coefficient  $C$  is defined as  $K/(\text{en-})$

TABLE II

*Proteolytic Activity of Partially Purified Lymphopeptidase*

Enzyme concentration, 0.009 mg of protein nitrogen per cc of test solution, pH 7.8 to 8.0

Substrate	Time	H <sub>2</sub> drolysis	
		No activator added	0.001M MnSO <sub>4</sub> present
	hrs	per cent	per cent
L-Leucylglycylglycine	1	25	26
	2	49	53
	4	91	90
	6	98	99
Diglycylglycine	1	23	21
	2	44	40
	4	81	
	8	97	
L-Alanylglycylglycine	1	24	24
	2	47	46
	4	86	
	8	99	
L-Leucinamide	6	1	2
Glycyl-L proline	2	0	1
	6	2	7

zyme concentration (expressed as mg of protein nitrogen per cc of test solution))

Calculation of the proteolytic coefficients of the crude saline extract of thymus and of the partially purified lymphopeptidase solution shows a 33-fold increase in  $C_{GGG}$  (from 1.2 to 40). The increase in  $C_{LGG}$  is somewhat less (25-fold), which may be attributed to the presence in the crude extract of some active leucine aminopeptidase which is eliminated in the course of the purification.

Preliminary experiments have shown that it is possible to concentrate lymphopeptidase further by adsorption at pH 6 on  $\text{C}\gamma$  alumina, followed by elution with M/15 phosphate buffer at pH 7.4. Fractional precipitation

TABLE III

*Kinetics of Hydrolysis of L-Leucylglycylglycine and of Diglycylglycine by Lymphopeptidase*

The pH was maintained at 7.9 in all cases

Enzyme concentration, protein N per cc test solution	Time	L Leucylglycylglycine			Diglycylglycine		
		Hydrolysis	$K^0_{LGG}$	$C^0_{LGG}$ *	Hydrolysis	$K^0_{GGG}$	$C^0_{GGG}$ *
mg	min	per cent			per cent		
0.0045	60	12	0.20		10	0.17	
	90	20	0.22		15	0.17	
	120	25	0.21	47	21	0.18	39
	180	38	0.21		32	0.18	
0.009	60	25	0.42		22	0.37	
	90	37	0.41	46	34	0.38	41
	120	49	0.41		44	0.37	
0.0135	30	19	0.63		17	0.57	
	60	38	0.63	47	31	0.52	40
	90	58	0.64		47	0.52	
0.018	30	26	0.87		23	0.77	
	60	51	0.85	48	46	0.77	43
0.027	30	38	1.27		32	1.07	
	60	75	1.25	47	63	1.05	39

\*  $C^0$  = The average of  $K^0$  per mg of protein nitrogen

TABLE IV

*pH Dependence of Lymphopeptidase Action*

Enzyme concentration, 0.10 mg of protein nitrogen per cc of test solution

pH	$K^0$	
	L Leucylglycylglycine	Diglycylglycine
6.8	0.35	0.28
7.3	0.38	
7.5		0.34
7.6	0.41	
7.8	0.43	0.38
8.0	0.45	0.39
8.1		0.37
8.2	0.42	
8.4	0.40	0.32
8.7	0.35	

with ammonium sulfate has given enzyme solutions with proteolytic coefficients ( $C_{GGG}$ ) of 85 to 102

*pH Dependence of Lymphopeptidase Action*—The pH optimum for the action of lymphopeptidase on L-LGG or GGG is near pH 8 (cf Table IV)

*Specificity of Lymphopeptidase*—As will be noted from Table II, partially purified lymphopeptidase hydrolyzes not only L-LGG and GGG but also L-alanylglycylglycine (L-AGG), and the rates of hydrolysis of these tripeptides are quite similar. It was reported previously (1) that the LGG-splitting enzyme in extracts of skin and in serum is readily inactivated at pH values below 5. A similar behavior is exhibited by lymphopeptidase, as is shown in Table V. In addition, following partial inactivation of the enzyme, the activity toward L-LGG, GGG, and L-AGG decreases in a parallel manner, thus supporting the view that the hydrolysis of the three tripeptides is effected by the same enzyme. It would appear, therefore, that the specificity requirements of lymphopeptidase permit of some variation in the nature of the amino acid residue which bears the free amino group.

The extent of hydrolysis of the tripeptides by lymphopeptidase does not

TABLE V  
*pH Stability of Lymphopeptidase*

The pH of the enzyme solution was adjusted by the addition of suitable amounts of 0.1 N hydrochloric acid to the veronal buffer-enzyme mixture. After 1 hour at 40°, the pH was readjusted to pH 7.8 and the appropriate substrate was added. Enzyme concentration, 0.009 mg of protein nitrogen per cc of test solution.

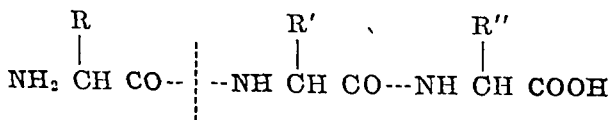
pH of enzyme solution	K°		
	L-Leucylglycylglycine	Diglycylglycine	L-Alanylglycylglycine
7.4	0.40	0.36	0.39
6.3	0.35	0.29	0.32
5.4	0.15	0.13	0.13
4.7	0.03	0.01	0.02
2.6	0.00	0.00	0.00

exceed that expected for the hydrolysis of one peptide bond (*cf* Table II). It has been shown previously (4, 1) that partially purified extracts of intestinal mucosa and of skin hydrolyze L-LGG at the peptide linkage adjacent to the free amino group. Lymphopeptidase also acts at this peptide linkage in L-LGG and hydrolyzes L-AGG at the analogous bond between the alanine and middle glycine residues. This is readily demonstrated by polarimetric observation of the course of hydrolysis, since the final rotation corresponds closely to the value for the mixture of the appropriate optically active amino acid and glycylglycine. In the case of L-LGG (0.05 M), after 100 per cent hydrolysis of one peptide linkage at pH 7.8, the reading was  $\alpha_D = -0.04^\circ$  (1 dm tube, 25°), while a mixture of L-leucine (0.05 M), glycylglycine (0.05 M), enzyme (0.008 mg of protein nitrogen per cc), and veronal buffer gave a rotation of  $-0.05^\circ$ . On the other hand, a comparable mixture of L-leucylglycine (0.05 M), glycine (0.05 M), enzyme, and buffer

gave a reading of  $+0.55^\circ$ . Similarly, for 100 per cent hydrolysis of one peptide linkage of L-AGG (0.05 M) at pH 7.8, the observed rotation was  $+0.01^\circ$ , and the same value was obtained for a mixture of L-alanine, glycylglycine, enzyme, and buffer. A mixture of L-alanylglycine, glycine, enzyme, and buffer gave a reading of  $+0.21^\circ$ . It may be concluded, therefore, that lymphopeptidase hydrolyzes L-LGG and L-AGG at the peptide linkage adjacent to the free amino group, and presumably splits the analogous peptide bond of other tripeptides which serve as substrates.

The data in Table VI show that the substitution of the free amino group of a substrate of lymphopeptidase, as in carbobenzoxy-L-LGG or carbobenzoxy-GGG, completely abolishes enzyme action. Since the action of lymphopeptidase is to hydrolyze LGG at the peptide bond between the leucine and middle glycine residues, this result suggests that the enzyme belongs to the group of aminopeptidases (5). Dipeptides such as glycylglycine, L-leucylglycine, or glycyl-L-leucine are not hydrolyzed to an appreciable extent, and it would appear that the presence of a free carboxyl group in adjacency to the sensitive peptide bond exerts an inhibitory effect on the enzymatic action.

The specificity of lymphopeptidase differs from that of previously characterized exopeptidases (aminopeptidase, carboxypeptidase), since it involves structural requirements in addition to a terminal free amino group and the peptide bond which is hydrolyzed. This is shown by the fact that glycylglycinamide and diglycylglycinamide are resistant to enzyme action, thus indicating the need for the presence, in a tripeptide, of an amino acid bearing a free  $\alpha$ -carboxyl group. Of especial interest in this connection is the failure of lymphopeptidase to hydrolyze, at an appreciable rate, such tetrapeptides as triglycylglycine, diglycyl-L-leucylglycine, or diglycyl-L-glutamylglycine. It may be concluded, therefore, that lymphopeptidase is an enzyme which acts as an "aminoexotripeptidase" and which requires in its substrates the groups indicated in the following formula by means of bold-faced letters:



It has been shown previously (6) that L-alanylsarcosylglycine is resistant to the action of the peptidase from intestinal mucosa which hydrolyzes L-AGG. A similar result was noted with lymphopeptidase (*cf* Table VI), and this indicates the need for the "peptide hydrogen" in the peptide bond which is split by the enzyme. The requirement for the presence of a hydrogen atom in the sensitive peptide bond may be interpreted as evidence for the view, expressed earlier (7), that, in the case of several proteolytic en-

zymes, the peptide bond which is hydrolyzed undergoes enolization in the course of the enzymatic action. An exception to this conclusion is the highly specific prolidase which hydrolyzes peptide bonds involving the imino group of proline or hydroxyproline (6, 4)

If GGG is modified so as to insert a methyl group in place of the peptide hydrogen of the peptide linkage adjacent to the free carboxyl group, the

TABLE VI  
*Specificity of Lymphopeptidase*

The rates of hydrolysis are given in terms of proteolytic coefficients, since the more resistant substrates required larger enzyme concentrations for accurate determination of the extent of hydrolysis

Substrate	pH	C <sup>o</sup>
Diglycylglycine	7.9	41
Glycylglycine	7.9	0
Glycylglycinamide	8.0	0.2
Diglycylglycinamide	7.6	0.3
Triglycylglycine	7.7	0.7
Glycylglycylsarcosine	7.9	27
Glycylglycyl-L-proline	7.9	29
Carbobenzoyldiglycylglycine	7.6	0
L-Alanylglycylglycine	7.8	43
D-Alanylglycylglycine	7.6	0.1
L-Alanylsarcosylglycine	8.0	0
L-Leucylglycylglycine	7.9	46
D-Leucylglycylglycine	7.7	0
L-Leucylglycine	8.0	0.1
Carbobenzoyl-L-leucylglycylglycine	7.8	0
Glycyl-L-leucylglycine	7.9	40
Glycyl-D-leucylglycine	7.9	0.1
Glycyl-L-leucine	7.9	0.1
Glycylglycyl-L-leucine	7.7	16.5
Glycylglycyl-D-leucine	7.6	3.8
Glycylglycyl-L-leucylglycine	7.6	0.8
Glycylglycyl-L-glutamylglycine	7.8	0.2
Glutathione	8.0	0.1

\* C<sup>o</sup> = the average K<sup>o</sup> per mg. of protein nitrogen

resulting glycylglycylsarcosine is readily hydrolyzed by lymphopeptidase. Similarly, glycylglycyl-L-proline also is split by the enzyme. It would appear, therefore, that enolization of the peptide bond adjacent to the free carboxyl group is not essential for lymphopeptidase action. This result is consonant with the fact that lymphopeptidase hydrolyzes tripeptides only at the peptide bond next to the free amino group.

The data in Table V show that D-LGG and D-AGG are resistant to en-



zyme action, thus giving evidence of stereochemical specificity with respect to the amino acid residue bearing the free amino group. Furthermore, glycyl-D-leucylglycine is not split appreciably by lymphopeptidase, in contrast to the behavior of the corresponding L peptide. In addition, glycyl-glycyl-D-leucine is hydrolyzed much more slowly than its L isomer, thus showing that the configuration of the amino acid bearing the free carboxyl group is also decisive for lymphopeptidase action. This stereochemical specificity with respect to the terminal amino acid at the carboxyl end of the tripeptide is in accord with the view that, in the hydrolysis of the sensitive peptide bond, the free carboxyl group is essential for the proper mutual

TABLE VII

*Effect of Added Substances on Lymphopeptidase Activity*

Enzyme concentration, 0.01 mg of protein nitrogen per cc of test solution, pH 7.8 to 7.9

Added substance	K°	
	L-Leucylglycylglycine	Diglycylglycine
None	0.44	0.39
0.01 M cysteine	0.42	0.38
0.005 M cyanide	0.43	0.38
0.005 " sulfide	0.42	0.39
0.001 " iodoacetate	0.45	0.40
0.001 " MnSO <sub>4</sub>	0.43	0.37
0.001 " ZnSO <sub>4</sub>		0.37
0.001 " MgSO <sub>4</sub>		0.40
0.001 " CoCl <sub>2</sub>		0.40
0.001 " NiSO <sub>4</sub> (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>		0.38
0.001 " Al <sub>2</sub> SO <sub>4</sub> K <sub>2</sub> SO <sub>4</sub>		0.38
0.001 " CdSO <sub>4</sub>		0.16

alignment of the substrate molecule and the "active center" of the enzyme (8).

The data presented above indicate that an exopeptidase may require, in its substrates, not only a terminal  $\alpha$ -amino or  $\alpha$ -carboxyl group in adjacency to the sensitive peptide bond but also additional polar groups at suitable positions in its substrate. Although lymphopeptidase appears to be the first instance of such an enzyme, the possibility exists that other cases of this kind may be found in the course of further investigation.

*Other Properties of Lymphopeptidase*—The action of lymphopeptidase is not inhibited appreciably by the presence of 0.01 M cysteine, 0.005 M cyanide, 0.0005 M sulfide, or 0.001 M iodoacetate (cf Table VII). As noted in Table II, the addition of manganese ions does not influence markedly the

enzymatic action of purified lymphopeptidase on GGG, although, in crude thymus extracts, the presence of  $MnSO_4$  causes a slight inhibition of the hydrolysis of this substrate. A number of metal ions other than manganese were also tested for their effect on the rate of hydrolysis of GGG by lymphopeptidase (cf Table VI). Of these, only the presence of cadmium ions resulted in a change in the rate, under the conditions of these experiments. The evaluation of this finding must await the results of further studies.

#### DISCUSSION

Attention has been drawn (9) to the similarity in the properties of the "aminopeptidase" of intestinal mucosa, which has been extensively purified by Ågren (10), and the tripeptide-splitting enzymes found in extracts of skin, lung, and muscle and in serum and lymph. The available data on the specificity, pH stability, and activation behavior of the comparable enzymes from these various sources are analogous to those described above for lymphopeptidase. It would appear to be a justifiable working hypothesis, therefore, to consider all these enzymatic actions to be due to an enzyme derived from lymphoid cells, although further comparison of highly purified preparations of the tripeptidases from various tissues is necessary before this view can be asserted with confidence. The results obtained to date, however, give added emphasis to the concept developed at the turn of the century by Metchnikoff (11) and others which assigns to lymphoid cells a significant rôle in the proteolytic mechanisms of body tissues and fluids. The manner in which the tripeptide-splitting lymphopeptidase may participate in protein metabolism is a problem for future study. It has already been suggested (12) that the intestinal peptidase which hydrolyzes L-AGG is identical with the "intrinsic factor" of Castle.

Examination of the available data (1, 4) also shows that crude extracts of all the various tissues mentioned above contain, in addition to tripeptidase activity, leucine aminopeptidase, and proliadase. The parallel occurrence of these three enzymes in a variety of tissues may be taken to suggest their common origin in lymphoid cells.

#### EXPERIMENTAL

*Partial Purification of Lymphopeptidase*—600 gm of calf thymus were minced with 1.2 liters of 2 per cent sodium chloride solution in a Waring blender, 50 cc of toluene were added, and the mixture was stirred mechanically for 2 hours at room temperature. The suspension was strained through fine cheese-cloth to give the crude extract used for the experiments reported in Table I.

The crude extract (1460 cc) was diluted with 4 volumes of water and heated to 50° for 1 hour with vigorous mechanical stirring. The suspension

was filtered by gravity in the cold room through fluted filters. To the clear filtrate (4.6 liters) there were added, with mechanical stirring, 1115 gm of ammonium sulfate. The precipitate which separated was removed by suction with the aid of Hyflo Super-Cel. To the filtrate (4.94 liters), 1015 gm of ammonium sulfate were added with stirring. The precipitate was collected by filtration through hardened filter paper. The moist filter cake weighed 6.5 gm.

To prepare a solution of lymphopeptidase, 1 gm of the filter cake was dissolved in 50 cc of water and dialyzed in a rocking dialyzer against distilled water for 72 hours at 4°. The precipitate which separated during dialysis was removed by filtration through fluted filter paper. A few drops of toluene were added as a preservative. If kept in the refrigerator when not in use, the enzyme solution retains its lymphopeptidase activity for 2 to 3 weeks.

*Measurement of Enzyme Activity*—In all cases, the concentration of the synthetic substrates was 0.05 mm per cc of test solution. The pH was maintained by means of 0.02 M veronal buffer at pH 6.8 to 8.7 and with 0.02 M citrate buffer at pH 5.0 to 5.4. The flasks containing substrate, buffer, and enzyme were kept in a water thermostat at 39°. The rate of hydrolysis was followed by measurement of the liberation of carboxyl groups by the method of Grassmann and Heyde (13). The data in Tables I to VI are given in terms of percentage of the carboxyl groups expected from the complete hydrolysis of one peptide linkage.

#### *Glycylglycylsarcosine*

*Carbobenzoxyglycylglycylsarcosine Benzyl Ester*—To an ethereal solution of sarcosine benzyl ester (prepared in the usual manner from 2 gm of the hydrochloride), there was added a solution of 2 gm of carbobenzoxyglycylglycinazide in ethyl acetate. On standing overnight at room temperature, 1.4 gm of the coupling product separated, m.p., 110–111°. The filtrate was washed with dilute hydrochloric acid, dilute bicarbonate solution, and water, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to a small volume under reduced pressure. A second crop (0.2 gm) of the product was obtained by the addition of petroleum ether.

$\text{C}_{20}\text{H}_{25}\text{O}_6\text{N}_3$  (427.4) Calculated, N 9.8, found, N 9.6

*Glycylglycylsarcosine*—1 gm of the carbobenzoxytripeptide benzyl ester was hydrogenated in methanol in the presence of palladium black. The peptide separated during the hydrogenation and was dissolved by the addition of water. The filtrate from the catalyst was evaporated, and on addition of alcohol, the peptide crystallized. Yield, 0.35 gm. The substance was dried at 100° *in vacuo* over phosphorus pentoxide.

$\text{C}_7\text{H}_{13}\text{O}_4\text{N}_2$	Calculated	C 41.4, H 6.4, N 20.7
203.2	Found	" 41.5, " 6.3, " 20.5

*Glycylglycyl-L-leucine*

*Carbobenzoxylglycylglycyl-L-leucine Methyl Ester*—To a solution of L-leucine methyl ester (prepared from 2.5 gm of the hydrochloride) in ethyl acetate, there were added 2 gm of carbobenzoxylglycylglycinazide. The reaction mixture was left at room temperature overnight and then washed with dilute hydrochloric acid, dilute bicarbonate solution, and water. The ethyl acetate solution was dried over  $\text{Na}_2\text{SO}_4$  and concentrated to a small volume under reduced pressure. The addition of ether and petroleum ether gave a crystalline precipitate. After recrystallization from ethyl acetate-petroleum ether, the substance melted at 93–94°. Yield, 1.4 gm.

$\text{C}_{19}\text{H}_{27}\text{O}_6\text{N}_3$  (393.4) Calculated, N 10.7, found, N 10.4

*Glycylglycyl-L-leucine*—1 gm of the carbobenzoxyltripeptide ester was dissolved in 25 cc of methanol, and 3.8 cc of  $\text{N NaOH}$  were added. After 20 minutes, the solution was acidified and concentrated under reduced pressure. The carbobenzoxyltripeptide which crystallized was collected, washed with water, and dried over phosphorus pentoxide. It was then hydrogenated in the usual manner to yield 0.5 gm of the desired tripeptide. For analysis, the product was recrystallized from ethanol-water and dried *in vacuo* at 100° over phosphorus pentoxide.

$\text{C}_{10}\text{H}_{19}\text{O}_4\text{N}_3$  Calculated C 49.0, H 7.7, N 17.1  
245.3 Found " 49.2, " 7.5, " 16.9  
 $[\alpha]_D^{25} = -28.0^\circ$  (2% in water)

*Glycylglycyl-D-leucine*

*Carbobenzoxylglycylglycyl-D-leucine Methyl Ester*—This compound was prepared in the same manner as was the L form, m p, 95–96°.

$\text{C}_{19}\text{H}_{27}\text{O}_6\text{N}_3$  (393.4) Calculated, N 10.7, found, N 10.5

*Glycylglycyl-D-leucine*—This compound was prepared from the above carbobenzoxyltripeptide ester in the same manner as was the L form.

$\text{C}_{10}\text{H}_{19}\text{O}_4\text{N}_3$  Calculated C 49.0, H 7.7, N 17.1  
245.3 Found " 49.1, " 7.7, " 17.0  
 $[\alpha]_D^{25} = +27.5^\circ$  (2% in water)

*Glycyl-D-leucylglycine*—This compound was prepared in the same manner as was the L form (14).

$\text{C}_{10}\text{H}_{19}\text{O}_4\text{N}_3$  Calculated C 49.0, H 7.7, N 17.1  
245.3 Found " 49.2, " 7.5, " 16.9  
 $[\alpha]_D^{25} = +42.6^\circ$  (2% in water)

*Diglycylglycinamide Acetate*

*Carbobenzoxylglycylglycine Ethyl Ester*—This compound was prepared by the coupling of carbobenzoxylglycylglycinazide (1.5 gm) with glycine ethyl

ester (prepared from 2.5 gm of the hydrochloride) by the procedure described above for carbobenzoxyglycylglycyl-L-leucine methyl ester. Yield, 1.4 gm, m p, 165°

$C_{16}H_{21}O_6N_3$  (351.3) Calculated, N 12.0, found, N 11.9

*Carbobenzoxydiglycylglycinamide*—1 gm of the above ester was dissolved in 25 cc of methanol previously saturated with dry ammonia at 0°, and the solution was allowed to stand at room temperature for 2 days. During this period, the amide separated from the solution. Yield, 0.9 gm. The substance was recrystallized from hot water, m p, 220°

$C_{14}H_{18}O_6N_4$  (322.3) Calculated, N 17.4, found, N 17.1

*Diglycylglycinamide Acetate*—0.5 gm of the carbobenzoxytripeptide amide was hydrogenated in methanol containing 10 cc of glacial acetic acid. On concentration of the filtrate, the product crystallized. It was recrystallized from absolute alcohol. Yield, 0.3 gm

$C_6H_{12}O_3N_4$	$C_2H_4O_2$	Calculated	C 38.7, H 6.5, N 22.6
248.2		Found	" 39.0, " 6.4, " 22.3

#### SUMMARY

Calf thymus extracts contain a peptidase which hydrolyzes tripeptides at the peptide linkage adjacent to the free amino group of the substrate. This enzyme, named lymphopeptidase, has been purified appreciably by fractional precipitation with ammonium sulfate and by dialysis against water. The specificity of the purified lymphopeptidase has been studied by means of a series of peptides and peptide derivatives closely related to the substrates for this enzyme, and the results indicate that it should be classified as an "aminoexotripeptidase." The fact that the sera as well as the extracts of various tissues from several animals exhibit peptidase activity closely similar to that of lymphopeptidase suggests that this enzyme is largely responsible for the tripeptide-splitting activity of these sera and tissue extracts.

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# PREPARATION OF D- AND L-METHIONINE FROM DL-METHIONINE BY ENZYMATIC RESOLUTION\*

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It has been shown previously (1, 2) that the synthesis of CO-NH linkages by papain as well as by other proteinases is characterized by extreme stereochemical specificity. Thus, upon the addition of cysteine-activated papain to a mixture of carbobenzoxy-DL-glutamic acid and aniline, there is formed carbobenzoxy-L-glutamic acid anilide, which separates in crystalline form. The carbobenzoxy-D-glutamic acid which remains in solution may then be hydrogenated to yield D-glutamic acid (2).

As noted elsewhere (3), it appears desirable to examine the feasibility of this enzymatic resolution method for the preparation of the optically active isomers of several amino acids (methionine, threonine, valine, and isoleucine) whose DL forms are now readily accessible by synthesis. In the present communication there is described the preparation, by the enzymatic resolution of DL-methionine, of D- and of L-methionine. A method for the resolution of DL-methionine has been described previously by Windus and Marvel (4), who used the classical procedure of Emil Fischer. Duschinsky and Jeannerat (5) prepared L-methionine from the racemate by the selective oxidation of the D isomer with D-amino acid oxidase.

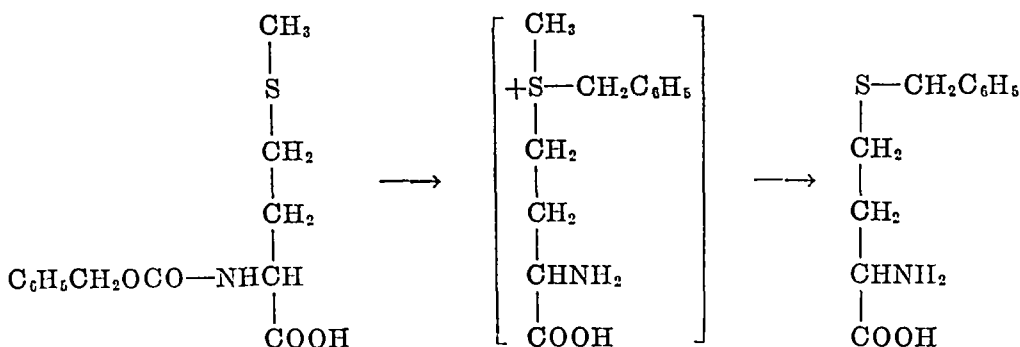
When carbobenzoxy-DL-methionine is incubated with aniline in the presence of cysteine-activated papain, carbobenzoxy-L-methionine anilide crystallizes with a yield of 95 per cent. From the filtrate, there may be isolated carbobenzoxy-D-methionine which, on catalytic hydrogenation, is converted to D-methionine of satisfactory purity.

It was intended to obtain L-methionine by the hydrolysis of carbobenzoxy-L-methionine anilide with hydrochloric acid. The main product of the cleavage was found to be not L-methionine, however, but S-benzyl-L-homocysteine. The formation of this compound from carbobenzoxy-L-methionine anilide may be explained by assuming the formation of benzyl chloride in the course of the acid hydrolysis of the carbobenzoxy group, followed by the conversion of the thioether to a sulfonium ion. The preferential elimination of the methyl group during the decomposition of the

\* This study was aided by grants from the American Cancer Society (on recommendation of the Committee on Growth of the National Research Council) and from the Rockefeller Foundation.



sulfonium ion would then lead to the formation of S-benzyl-L-homocysteine



Support for this interpretation comes from the observation that, if DL-methionine is refluxed with benzyl chloride and hydrochloric acid, S-benzyl-DL-homocysteine is formed

The application of the enzymatic resolution method thus offers an additional method for the preparation of S-benzyl-L-homocysteine. This compound was first obtained by du Vigneaud and Patterson (6) from DL-methionine by the conversion of the latter substance to DL-homocysteine, which was then reduced with sodium in liquid ammonia. Treatment with benzyl chloride gave S-benzyl-DL-homocysteine which was resolved by fractional crystallization of the brucine salts of the N-formyl derivatives. Du Vigneaud and Patterson prepared D- and L-homocysteine from the optically active S-benzyl derivatives by treatment with sodium in liquid ammonia.

The difficulty encountered in the attempted preparation of L-methionine from carbobenzoxy-DL-methionine may be avoided if, in place of the carbobenzoxy derivative, the benzoyl compound is employed in the enzymatic resolution. In the presence of papain, benzoyl-L-methionine anilide is formed in 97 per cent yield and pure L-methionine may be prepared by acid hydrolysis of this derivative. The isolation of pure benzoyl-D-methionine from the filtrate of the anilide proved to be difficult, in contrast to the situation found in the case of the corresponding carbobenzoxy compound. Hydrolysis of the impure benzoyl-D-methionine gave partially racemic preparations of the amino acid.

The ready availability of the optically active isomers of methionine makes possible their use in the synthesis of peptides of L- and of D-methionine. The preparation of such peptides will be the subject of a subsequent communication.

#### EXPERIMENTAL

*Carbobenzoxy-DL-methionine*—To a solution of 30 gm (0.2 mole) of DL-methionine in 100 cc of 2 N NaOH, there were added 37.5 gm (0.22 mole)

of carbobenzoxy chloride and 120 cc of 2 N NaOH in five portions over a period of 30 minutes. The reaction mixture was kept at 0° and stirred continuously during this time. After further stirring for 1 hour at room temperature, the solution was acidified to Congo red with concentrated hydrochloric acid, causing the precipitation of crystalline carbobenzoxy-DL-methionine. The crystals were collected and dried over phosphorus pentoxide *in vacuo*. Yield, 50 gm (88 per cent), m p, 110–112°. After recrystallization from 40 per cent ethanol, the substance melted at 112°.

$C_{12}H_{17}O_4NS$	Calculated	C 55.1, H 6.1, N 4.9
283.2	Found	" 55.2, " 6.0, " 4.9

*Enzymatic Synthesis of Carbenzoxy-L-methionine Anilide*—14.5 gm (0.05 mole) of carbobenzoxy-DL-methionine were dissolved in 50 cc of N NaOH and added to 9.5 cc (0.11 mole) of aniline. A solution of 0.6 gm of cysteine hydrochloride in 20 cc of water was added, followed by 40 cc of 0.2 M citrate buffer (pH 5.0). A solution obtained by the extraction of 3 gm of crude papain (dried papaya latex) with 40 cc of water was then introduced and the reaction mixture was diluted to 250 cc with water. Separation of the anilide began immediately, crystallization being induced by vigorous shaking of the flask. The reaction mixture was placed in a constant temperature bath at 38° for 4 days. The anilide which separated was removed daily and the filtrate was replaced in the bath after adjusting the pH to 5 with a few drops of concentrated hydrochloric acid. The combined precipitates weighed 8.7 gm (95 per cent). The compound was recrystallized from 40 per cent ethanol, m p, 162.5°.

$C_{19}H_{21}O_4N_2S$	Calculated	C 63.6, H 6.2, N 7.8
358.3	Found	" 63.5, " 6.2, " 8.1
$[\alpha]_D^{25} = -14.8^\circ$ (1.45% in glacial acetic acid)		

*Carbenzoxy-D-methionine*—The filtrate remaining from the enzymatic synthesis of carbobenzoxy-L-methionine anilide was heated to boiling to coagulate the proteins and was decolorized with a few gm of Darco. The clear filtrate was acidified to Congo red with concentrated hydrochloric acid. The syrup which separated crystallized after being kept in the ice-box for 24 hours. The crystals were dissolved in ether and the ethereal solution was extracted with an aqueous solution of potassium bicarbonate. Upon acidification of the aqueous layer, there separated crystalline carbobenzoxy-D-methionine which was collected and washed with cold water. Yield, 7 gm (85 per cent). After recrystallization from 50 per cent ethanol, the substance melted at 69–70°.

$C_{12}H_{17}O_4NS$	Calculated	C 55.1, H 6.1, N 4.9
283.2	Found	" 54.9, " 6.1, " 4.8

*D-Methionine*—2.85 gm (0.01 mole) of carbobenzoxy-D-methionine were dissolved in 20 cc of methanol containing a few drops of glacial acetic acid and hydrogenated with palladium black as the catalyst. The hydrogenation required 11 hours and fresh catalyst was added at the end of 5 hours. 50 cc of hot water were added to dissolve the free amino acid, and the catalyst was removed by filtration and washed on the filter with more hot water. The filtrate and washings were concentrated to dryness *in vacuo* and the residue was extracted with ether to remove any unchanged carbobenzoxy-D-methionine. The ether-insoluble material was recrystallized from 75 per cent ethanol. The crystalline D-methionine was collected, washed with small amounts of absolute alcohol and ether, and dried over phosphorus pentoxide. Yield, 1.0 gm (67 per cent).

$C_8H_{11}O_2NS$	Calculated	C 40.2, H 7.5, N 9.4
149.2	Found	" 40.1, " 7.4, " 9.1
$[\alpha]_D^{27} = -21.5^\circ$ (1.2% in 0.2 N hydrochloric acid)		

Windus and Marvel (4) report  $[\alpha]_D^{25} = -21.18^\circ$  (0.8 per cent in 0.2 N hydrochloric acid).

*S-Benzyl-L-homocysteine*—2.5 gm (0.0067 mole) of carbobenzoxy-L-methionine anilide were refluxed with 20 cc of concentrated hydrochloric acid for 10 hours. The hydrolysate was extracted with ether and the aqueous layer was concentrated under reduced pressure. The concentrate was neutralized with saturated lithium hydroxide solution, yielding a heavy white precipitate which was collected and washed with small amounts of water, alcohol, and ether. The product was recrystallized from boiling water. Yield, 0.5 gm (30 per cent), m p, 243–244° (with decomposition). Du Vigneaud and Patterson (6) report 247–252° for S-benzyl-D-homocysteine.

$C_{11}H_{16}O_2NS$	Calculated	C 58.6, H 6.7, N 6.2
225.2	Found	" 58.6, " 6.7, " 6.1
$[\alpha]_D^{25} = +27.2^\circ$ (1% in N hydrochloric acid)		

Du Vigneaud and Patterson (6) found for S-benzyl-D-homocysteine  $[\alpha]_D^{25} = -25^\circ$  (1 per cent in N hydrochloric acid).

*S-Benzyl-DL-homocysteine*—1.5 gm (0.01 mole of DL-methionine) were dissolved in 20 cc of concentrated hydrochloric acid, 1.4 cc (0.012 mole) of benzyl chloride were added, and the mixture was refluxed for 11 hours. The reaction mixture, after being cooled, was extracted with ether, and the aqueous layer was concentrated to a syrup under reduced pressure. The residue was taken up in 30 cc of water and neutralized with saturated lithium hydroxide solution. The resulting precipitate was collected and washed successively with cold water, ethanol, and ether. Yield, 1.0 gm (45 per cent). After recrystallization from boiling water, the substance

melted with decomposition at 240–243° Du Vigneaud and Patterson (6) report 240–250°

$C_{11}H_{15}O_2NS$  (225.2) Calculated, N 6.2, found, N 5.9

*Benzoyl-DL-methionine*—This compound was prepared according to the directions of Steiger (7) for the benzylation of amino acids. 32 gm of the benzoyl derivative were obtained from 20 gm of DL-methionine, representing a yield of 95 per cent. The crude material melted at 145–150°. After recrystallization from 33 per cent ethanol, the compound melted sharply at 151°. This value agrees with that reported by Hill and Robson (8).

*Enzymatic Synthesis of Benzoyl-L-methionine Anilide*—19.45 gm (0.077 mole) of benzoyl-DL-methionine were dissolved in 77 cc of *N* NaOH and 13.9 gm (0.154 mole) of aniline were added. A solution of 0.93 gm of cysteine hydrochloride in 25 cc of water was then added, followed by 40 cc of 0.2 *M* citrate buffer (pH 5.0). The enzyme solution, prepared by extracting 4.6 gm of papain with 62 cc of water, was introduced, and the mixture was agitated and placed in a constant temperature bath at 38°. Another 190 cc of buffer and more water were added gradually over a period of 2 hours, making the total volume 750 cc. After 18 hours, the benzoyl-L-methionine anilide which had separated was collected and the filtrate was replaced in the bath. The first crop weighed 11.3 gm (90 per cent). Another 0.9 gm was collected after the next 24 hours, giving a total yield of 12.2 gm (97 per cent). The compound was purified by recrystallization first from ethyl acetate and then from ethanol-water, *m p*, 159°.

$C_{15}H_{20}O_2N_2S$	Calculated	C 65.8, H 6.2, N 8.5
328.3	Found	" 65.8, " 6.2, " 8.2

*Acid Hydrolysis of Benzoyl-L-methionine Anilide*—2.2 gm (0.0067 mole) of benzoyl-L-methionine anilide were suspended in 150 cc of 6 *N* hydrochloric acid and refluxed on a sand-bath for 12 hours. After cooling the mixture, the benzoic acid which had crystallized was removed by filtration. The filtrate was concentrated under reduced pressure to 50 cc and then was extracted with two 35 cc portions of ether. The aqueous layer was concentrated and the residue was taken up in 15 cc of absolute alcohol. The addition of 5 cc of pyridine caused the crystallization of L-methionine. The crystals were collected and washed successively with absolute alcohol and ether. Yield, 0.5 gm (50 per cent). The rotation of the unrecrystallized material was  $[\alpha]_D^{24} = +20.7^\circ$  (1 per cent in 0.2 *N* hydrochloric acid). After recrystallization from 75 per cent ethanol, the optical activity was unchanged. The substance melted at 281° (corrected) with previous darkening and shrinkage. Mueller (9) reports a melting point of 283° for L-methionine.

$C_6H_{11}O_2NS$	Calculated	C 40.2, H 7.5, N 9.4
149.2	Found	" 40.0, " 7.3, " 9.1

*Acid Hydrolysis of Benzoyl-D-methionine*—The filtrate from the enzymatic synthesis of benzoyl-L-methionine anilide was heated to boiling to coagulate the proteins, was decolorized with Darco and filtered, and the filtrate was concentrated *in vacuo* to 185 cc. After acidification to Congo red, the benzoyl-D-methionine separated as an oil which crystallized readily upon stirring and scratching. The crude crystals were collected, washed with a small amount of cold water, and dried over phosphorus pentoxide and sodium hydroxide. Yield, 9.4 gm (96 per cent). Partial purification was effected by the extraction of an ethyl acetate solution of this material with aqueous potassium bicarbonate, and acidification of the bicarbonate solution. The crystalline material which resulted was washed and dried as before. Further purification by recrystallization was unsuccessful because the solubility of benzoyl-D-methionine proved to be greater than that of benzoyl-DL-methionine. Therefore, 2.55 gm (0.01 mole) of the partially purified benzoyl-D-methionine were suspended in 250 cc of 10 per cent hydrochloric acid and refluxed on a sand-bath for 12 hours. The benzoic acid which had separated was removed by filtration, and the filtrate was concentrated under reduced pressure to a volume of 125 cc. After extraction with two 60 cc portions of ether, the solution was further concentrated *in vacuo* and the residue was taken up in 18 cc of absolute alcohol. The alcoholic solution was clarified by filtration and the methionine was precipitated by the addition of 4 cc of pyridine. Yield, 1.0 gm (67 per cent). The optical rotation of the product was  $[\alpha]_D^{20} = -16.7^\circ$  (1.7 per cent in 0.2 N hydrochloric acid). Recrystallization of 0.9 gm of the above material from 100 cc of 75 per cent ethanol yielded 0.6 gm of methionine with a rotation of  $[\alpha]_D^{20} = -15.7^\circ$  (0.7 per cent in 0.2 N hydrochloric acid), thus indicating that the material was being enriched with respect to DL-methionine rather than D-methionine.

An additional fraction (0.16 gm) was recovered from the mother liquor, and this had a rotation of  $[\alpha]_D^{25} = -20.7^\circ$  (0.8 per cent in 0.2 N hydrochloric acid).

$C_8H_{11}O_2NS$	Calculated	C 40.2, H 7.5, N 9.4
149.2	Found	" 40.4, " 7.5, " 9.2

Although reasonably pure D-methionine was obtained, the procedure was less satisfactory than that involving the hydrogenation of carbobenzoxy-D-methionine.

#### SUMMARY

DL-Methionine has been resolved by the action of papain on a mixture of carbobenzoxy-DL-methionine and aniline. The anilide of the L isomer separates in crystalline form, and from the filtrate there may be isolated carbo-

benzoxy-D-methionine which upon catalytic hydrogenation is converted to D-methionine. Acid hydrolysis of carbobenzoxy-L-methionine anilide yields S-benzyl-L-homocysteine. L-Methionine may be prepared by the enzymatic synthesis of benzoyl-L-methionine anilide from benzoyl-DL-methionine and aniline, followed by acid hydrolysis of the anilide.

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# THE UTILIZATION OF PEPTIDES BY LACTIC ACID BACTERIA\*

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The extensive use of the lactic acid bacteria for the microbiological assay of amino acids has provided valuable data on the amino acid composition of proteins (1). In some cases, attempts have been made to determine the amino acid content of materials such as plasma (2), urine (3), and partial hydrolysates of tissue proteins (4) without reference to the question as to whether these materials contained peptides of the amino acid under test and whether these peptides were utilized by the test organism as effectively as the unsubstituted amino acid. Subsequent work (5) showed, indeed, that acid hydrolysis of such complex materials markedly raised their apparent amino acid content. The experiments described in the present communication indicate that there exists considerable variation in the utilization of leucine peptides by *Lactobacillus arabinosus* and by *Streptococcus faecalis*. Similar experiments have been reported recently by Ågren (6), who found differences in the utilization of various leucine and valine peptides (of unstated configuration) by several lactic acid bacteria. In addition, it has been shown previously (7) that the *leucineless* mutant of *Escherichia coli* exhibits considerable selectivity in the utilization of leucine peptides for growth. The suggestion has been made (7, 8) that, in the case of many peptides, utilization for growth is preceded by enzymatic hydrolysis to yield the essential amino acid. Differences in the effectiveness of such peptides in replacing an essential amino acid may be taken, therefore, to reflect differences in the rate of cleavage of the peptides by the bacterial peptidases.

Recent work has shown, however, that some peptides may be more effective in promoting bacterial growth than are the component amino acids. This has been brought out strikingly by the work of Woolley (9) on "strepogenin," and Womack and Rose (10) have raised the question as to the possible identity of this material with a peptide-like factor, which they have found to promote the growth of the rat. Moreover, data have been obtained<sup>1</sup> to show that, in the case of a *prolineless* mutant of *Escherichia coli*, certain proline peptides are more effective growth factors than is proline itself. It is clear, therefore, that there exist particular combinations of amino acids bound in peptide linkage which may be utilized in metabolism without prior enzymatic hydrolysis.

\* This study was aided by grants from the Sugar Research Foundation, Inc., and from the Rockefeller Foundation.

<sup>1</sup> Simmonds, S., and Fruton, J. S., unpublished experiments.



*Testing Methods*—The microorganisms used were *Lactobacillus arabinosus* 8014,<sup>2</sup> *Lactobacillus casei* 7469,<sup>2</sup> *Streptococcus faecalis* 9790,<sup>2</sup> and *Leuconostoc mesenteroides* P-60 8042.<sup>2</sup> Stock cultures of the organisms were carried in a medium containing 2 per cent Difco yeast extract, 0.5 per cent glucose, 2 per cent sodium acetate, and 1.5 per cent agar. The components of the basal medium differ for the most part only quanti-

TABLE I  
Composition of Complete Basal Medium

Component	Amount per 10 cc. final medium	Component	Amount per 10 cc. final medium
	mg		mg
Amino acid mixture		Salts C* (0.2 cc. per 10 cc. of final medium)	
L-Asparagine	4	MgSO <sub>4</sub> 7H <sub>2</sub> O	8.0
L-Glutamic acid	5	NaCl	0.4
L-Lysine HCl	2	FeSO <sub>4</sub> 7H <sub>2</sub> O	0.4
L-Cystine	1	MnSO <sub>4</sub> 4H <sub>2</sub> O	1.6
L-Arginine HCl	1	Glucose	200
L-Histidine HCl H <sub>2</sub> O	1	Purines and pyrimidines	γ
L-Proline	1	Adenine sulfate 2H <sub>2</sub> O	100
L-Tyrosine	1	Guanine HCl 2H <sub>2</sub> O	100
L-Serine	1	Uracil	100
L-Leucine†	1	Xanthine	100
DL-Tryptophan	1	Vitamins	
DL-Alanine	2	Biotin	0.05
DL-Threonine	2	Folic acid	0.05
DL-Isoleucine	2	p-Aminobenzoic acid	0.5
DL-Valine	2	Thiamine HCl	10.0
DL-Methionine	2	Riboflavin	10.0
DL-Phenylalanine	2	Pyridoxine HCl	10.0
Glycine	1	Nicotinic acid	10.0
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	30	Calcium pantothenate	10.0
Sodium acetate	200		
KH <sub>2</sub> PO <sub>4</sub>	25		
K <sub>2</sub> HPO <sub>4</sub>	25		

\* Roberts and Snell (11)

† Omitted in the assay for this amino acid and for leucine derivatives

tatively from those usually employed, but because uniform success has been attained with this medium for all of the lactic acid bacteria listed above, its composition is presented in Table I.

The procedure in conducting the microbiological assay is similar to that generally employed (12). Prior to use, the inocula were diluted 1:30 or 1:60, as indicated, and stock cultures were transferred to fresh agar each

<sup>2</sup> American Type Culture Collection, Georgetown University, Washington, D. C.

week All assay tubes were incubated at 37° and removed at suitable intervals for turbidimetric measurement with the Evelyn photoelectric colorimeter (filter No 620) or for the estimation of the acid production by titration with 0.1 N NaOH

*Utilization of Leucine Peptides by Lactic Acid Bacteria*—It has been reported previously (13) that D-leucine is not used by *Lactobacillus arabinosus* for growth (as measured by acid production after an incubation period of 72 hours) and that this amino acid does not inhibit the activity of L-leucine This result has been confirmed in the present studies and it has also been found that the same is true for *Streptococcus faecalis* (cf Table II) It was noted, however, that of two commercial preparations of DL-leucine only one gave growth responses which were to be expected on the basis of its L-leucine content The other preparation of DL-leucine consistently gave lower values and it may be concluded, therefore, that this material was impure<sup>3</sup> This finding further emphasizes the need for careful checking of the purity of the standards used for the microbiological assay of amino acids

In evaluating the utilization of the leucine peptides, it was desirable to estimate the extent of bacterial growth at different time intervals, in addition to the customary determination of the acid production after 72 hours This was done by measurement of turbidity after 16 and 40 hours, and the data in Table III represent the growth response to the addition of L-leucine In order to have a common basis for the comparison of the utilization of the leucine peptides, the concentration levels at which they were tested were such that the amount of leucine they could yield on complete hydrolysis fell within the range of the leucine concentrations given in Tables II and III The relative growth-promoting activity of each leucine derivative could then be expressed as per cent of that to be expected if all the leucine in the compound were available for growth In Table IV, the values for the "per cent leucine activity" are given as averages of duplicate determinations when these agreed within reasonable limits It will be noted that, in two cases, such agreement was not obtained

The data presented in Table IV show that all of the peptides of L-leucine which were tested served as growth factors for the two organisms employed in these studies It will be noted, however, that even at 72 hours utilization in the case of most of the peptides was less than that expected if all of the peptide-bound leucine were available for growth Moreover, there was found appreciable variation in the response of the two organisms to individual peptides such as glycyl-L-leucine Although certain of the peptides, e g, L-leucylglycylglycine, produced a similar growth response relative to

<sup>3</sup> A similar result was obtained with the *leucineless* mutant of *Escherichia coli* (Simmonds, S, and Fruton, J S, unpublished experiments)

TABLE II  
*Acid Production by Lactic Acid Bacteria in Presence of Leucine*

Leucine preparation added to basal medium	Amount per tube*	0.1 N acid formed per tube after 72 hrs	
		<i>Lactobacillus arabinosus</i>	<i>Streptococcus faecalis</i>
	$\gamma$	cc	cc
L-Leucine†	0	0 2	0 3
	20	3 4	2 4
	40	6 2	4 5
	60	8 4	6 4
	80	10 4	7 4
	100	12 6	8 0
" + D-leucine†	0	0 2	0 2
	20	3 3	2 3
	40	6 0	4 4
	60	8 3	6 2
	80	10 1	7 2
	100	12 2	8 1
DL Leucine‡	0	0 2	0 3
	20	3 6	2 1
	40	6 2	4 3
	60	8 4	6 1
	80	10 6	7 1
	100	12 6	7 9
" §	0	0 1	0 2
	20	0 8	0 8
	40	1 8	1 7
	60	3 2	2 6
	80	4 1	3 4
	100	4 9	4 4

\* Refers to amount of L-leucine added

† Obtained through the courtesy of Dr William H Stein

‡ Merck preparation

§ Eastman preparation.

TABLE III  
*Growth of Lactic Acid Bacteria in Presence of Leucine*

Amount of L leucine per tube	Galvanometer reading			
	<i>Lactobacillus arabinosus</i>		<i>Streptococcus faecalis</i>	
	16 hrs	40 hrs	16 hrs	40 hrs
$\gamma$				
0	94	93	98	94
20	83	73	88	83
40	74	56	80	74
60	64	42	72	66
80	58	36	65	58
100	51	32	61	51

the leucine standard at 16, 40, and 72 hours, in the case of other peptides, notably L-leucyl-L-tyrosine and diglycyl-L-leucylglycine, the time of incubation was an important factor in determining the extent of bacterial growth. These findings indicate that the growth-promoting activity of L-leucine peptides depends upon the position of the leucine residue with respect to the other amino acid residues, as well as upon the nature of the other amino acid residues. Furthermore, these results support the view that the rate of

TABLE IV  
Effect of Leucine Derivatives on Growth of Lactic Acid Bacteria

Compound*	Growth promoting activity†					
	<i>Lactobacillus arabinosus</i>			<i>Streptococcus faecalis</i>		
	16 hrs	40 hrs	72 hrs	16 hrs	40 hrs	72 hrs
	per cent	per cent	per cent	per cent	per cent	per cent
L-Leucylglycine	55	51	54	63	76	80
D-Leucylglycine		15	2		22	2
N-Methyl-DL-leucylglycine						
L-Leucylglycylglycine	63	66	76	58	62	57
D-Leucylglycylglycine		18	15		2	
L-Leucyl-L-tyrosine	71	86	102	92	106	93
L-Leucinamide acetate	13, 34	27	47	35	13	13
D-Leucinamide "						
Glycyl-L-leucine	103	99	102	63	66	72
Glycyl-D-leucine					17	
Diglycyl-L-leucylglycine		22	58	52	66	74
Triglycyl-L-leucylglycine‡		36	62			
Carbobenzoylglycyl-L-leucine		18, 42	68			
Acetyl-L-leucine			6			
Acetyldehydroleucine						
Acetyldehydroleucylglycine						
Acetyldehydroleucinamide						

\* The bibliographic references to the preparation of these compounds are given by Simmonds *et al.* (7)

† Expressed as per cent of that to be expected if all the leucine in the compound were available for growth. The blank indicates that no growth was noted at any concentration level tested.

‡ This substance was not tested with *Streptococcus faecalis*.

enzymatic hydrolysis of these peptides plays a significant rôle in their utilization by microorganisms.

In agreement with the results obtained with the leucineless mutant of *Escherichia coli*, the peptides containing D-leucine were uniformly inactive, regardless of the organism used, time of incubation, or peptide concentration. It is worthy of note that the leucine derivatives L-leucinamide and carbobenzoylglycyl-L-leucine are utilized by *Lactobacillus arabinosus*, and

only the first of these substances is at all effective for *Streptococcus faecalis*. In this connection, it should be of interest to compare the amino- and carboxypeptidase activity of these two organisms. Leucine derivatives such as N-methyl-DL-leucylglycine, acetyldehydroleucine, acetyldehydroleucylglycine, and acetyldehydroleucinamide were inactive for both organisms, and only slight activity was noted for acetyl-L-leucine in the case of *Lactobacillus arabinosus*.

"*Strepogenin*" *Activity of Peptides*—Sprince and Woolley have called attention (14) to the presence, in liver extracts and in partial hydrolysates of casein, of a peptide (strepogenin) which favors the growth of *Lactobacillus casei*, and Woolley has reported further (9) that a mixture of the diastereo-

TABLE V  
*Strepogenin Activity of Various Materials*

Component	Amount per 10 cc	Galvanometer reading*			
		<i>Lacto- bacillus arabi- nosus</i>	<i>Lacto- bacillus casei</i>	<i>Leuconos- toc mesen- teroides</i>	<i>Strepto- coccus faecalis</i>
	mg				
Basal medium		21	98	89	45
“ “ + liver fraction L	10	14	59	44	41
	5		75		
“ “ + L-serylglycyl-L-glu- tamic acid	8		82		
	3		90		
Basal medium + L-seryl-L-alanyl-L- glutamic acid	10		100		

\* Readings made with Evelyn photoelectric colorimeter after 18 hours incubation at 37°, inoculum dilution 1:60. Uninoculated control tubes were used as blanks for setting the galvanometer at 100.

isomeric D- and L-serylglycyl-L-glutamic acids has strepogenin activity. In the course of the present investigation, L-serylglycyl-L-glutamic acid was synthesized by the reaction of carbobenzoxy-L-serylglycinazide with L-glutamic acid diethyl ester, followed by the saponification of the carbobenzoxy-tripeptide ester and the removal of the carbobenzoxy group by catalytic hydrogenation with palladium black. In addition, L-seryl-L-alanyl-L-glutamic acid was made by an analogous procedure.<sup>4</sup>

Before testing the activity of these and other peptides for strepogenin activity, it appeared desirable to determine whether only *Lactobacillus casei* required strepogenin for growth. The data in Table V show that, of the four organisms tested, only *L. casei* and *Leuconostoc mesenteroides* P-60

<sup>4</sup> A detailed description of the synthesis of these tripeptides will be presented in a separate communication.

require a source of streptogenin, since liver fraction L<sup>5</sup> did not affect appreciably the growth of *L. arabinosus* or *Streptococcus faecalis*

It will be noted from Table V that L-serylglycyl-L-glutamic acid exhibits a slight but unmistakable growth-promoting activity for *Lactobacillus casei*. This confirms, in a qualitative sense, the result reported by Woolley. Since 1 mg of the tripeptide was equivalent in activity to about 0.5 mg of the liver fraction, it is clear that the latter must contain a substance which is considerably more active than the synthetic tripeptide. It may be added that the closely related L-seryl-L-alanyl-L-glutamic acid was inactive under the conditions of our assay, as were  $\alpha$ -L-glutamyl-L-glutamic acid,  $\alpha$ -L-glutamylglycylglycine, diglycyl- $\alpha$ -L-glutamylglycine, and glycyl- $\alpha$ -L-glutamyl-L-tyrosine.

The authors wish to express their thanks to Miss Barbara Illingworth for valuable assistance in the course of this investigation.

#### SUMMARY

Experiments on the utilization of various peptides of L-leucine by *Lactobacillus arabinosus* and *Streptococcus faecalis* have shown that the growth-promoting activity of such peptides depends on the position of the leucine residue with respect to the other amino acid residues, the nature of the other amino acids, and the time of incubation.

The streptogenin activity of L-serylglycyl-L-glutamic acid for *Lactobacillus casei*, reported by Woolley, has been confirmed.

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## THE SOLUBILITY OF NITROUS OXIDE IN BLOOD AND BRAIN\*

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Since the formulation of the lipid theory of anesthesia (1) there has been some interest in the solubilities of various volatile anesthetics in the brain (2-6). Unfortunately the results obtained *in vivo* have been equivocal and inexact because of the technical difficulties of obtaining and analyzing without loss samples of brain containing volatile gas. Even the studies on solubility *in vitro*, on which the theory was largely based, were made on peanut, olive, or similar oils rather than on brain lipides. Our attention was drawn to the problem of the solubility of gases in brain in the course of the development of a method for measurement of cerebral blood flow by means of the blood-brain exchange of an inert gas, nitrous oxide (7). It can be shown that cerebral blood flow may be calculated from the arterial (*A*) and cerebral venous (*V*) blood nitrous oxide concentrations over a time interval (*u*) measured from the onset of inhalation of a comparatively low tension of nitrous oxide. The interval *u* must be of sufficient length to insure practically complete equilibrium between brain and blood draining the brain with respect to mean nitrous oxide tension. If the results are to be expressed in flow per unit weight of brain rather than in terms of flow per unit of nitrous oxide capacity, a factor (*S*) must be introduced, representing the brain-blood partition coefficient of nitrous oxide or the ratio of nitrous oxide dissolved per gm. of brain to that dissolved per cc. of blood at a constant nitrous oxide tension and at 37°. The formula for calculation of cerebral blood flow, which is derived elsewhere may be expressed as follows

$$\text{Cerebral blood flow} = \frac{V_u S}{\int_0^u (A - V) dt}$$

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In order to make possible the calculation of absolute values it was necessary to evaluate the partition coefficient ( $S$ ) and the minimum time ( $u$ ) for equilibrium between the brain and its venous blood. This was attempted by means of both *in vitro* and *in vivo* techniques.

### Methods

*Determination of Nitrous Oxide Solubilities in Blood and Brain in Vitro*—Freshly shed, heparinized whole blood is used as such. The brain, however, is too viscous for convenient handling, a representative sample of about 10 gm is accurately weighed and homogenized with exactly 5 cc of distilled water in a Potter glass homogenizer (8). The homogenate is pressed through coarse gauze to remove small shreds of connective tissue and transferred to a 50 cc glass syringe in which equilibration with nitrous oxide is to occur. All air is expelled from the syringe which is then filled with pure nitrous oxide after several flushings with the same gas. The tip of the syringe is immediately closed by means of a sealed needle hub and the syringe containing the nitrous oxide and blood or brain homogenate is rotated slowly in a water bath maintained at 37°. It is important to make sure frequently that the plunger of the syringe is free to move in the barrel to compensate for temperature-induced volume changes and that the syringe is not more than a cm below the surface of the water of the bath. At intervals of 15 minutes the syringe is removed from the bath, the gas is expelled, and fresh nitrous oxide added for three such flushings. Equilibration is then allowed to proceed for another hour. At the end of that time, with the syringe held vertically in the bath, the gas is entirely expelled and the cap replaced. The syringe may now be removed from the bath and part of its contents analyzed for nitrous oxide as follows, after which the remainder may be reequilibrated. 5 drops of caprylic alcohol plus 3 cc of distilled water are extracted for 3 minutes in the 50 cc chamber of a Van Slyke-Neill manometric apparatus, then expelled as completely as possible, 1 cc of mercury in addition being permitted to rise into the cup. The water and caprylic alcohol are removed from the surface of the mercury in the cup. A stout capillary tube of about 2 mm bore and a cm longer than the depth of the cup on the manometric apparatus is now securely fastened to the tip of the syringe by means of a short length of heavy plastic tubing. The free end of this capillary tube, which is somewhat tapered to resemble the tip of an Ostwald-Van Slyke pipette, is fitted with a rubber tip for sealing against the bottom of the cup. The contents of the syringe are carefully expressed to this tip and a little excess is expelled. The capillary is then held vertically, its rubber tip pressed against the bottom of the cup, and, against a slight positive pressure of mercury in the manometric apparatus, the contents of the syringe, by pressure on the plunger, are carefully forced

into the chamber, stopping accurately at the 2 cc mark. The stop-cock at the top of the chamber is now closed, the cup emptied, and the mercury in the chamber brought down to the 50 cc mark. 6 cc of deaerated oxygen absorber (KOH-hydrosulfite-anthraquinone reagent employed in blood oxygen analyses (9)) are now added to the cup and the lower 5 cc admitted to the chamber in several quick additions to wash the 2 cc volume clean. The upper cock is sealed with mercury and the contents of the chamber are extracted for about 5 minutes. The liquid is then permitted to rise smoothly to the 2 cc mark and pressure and temperature readings are taken. Extraction at 50 cc is repeated for 5 minute periods until the pressure reading (corrected for any temperature change) remains constant for three successive determinations. Nitrous oxide concentration is calculated as follows

$$\text{vol } \% \text{ N}_2\text{O} = f_{\text{N}_2\text{O}}[r_a - (r_0 + C_w)]$$

where  $r_a$  = the manometric reading for the sample and  $r_0$  = the manometric reading for the blank. This is run once for each series of analyses, and consists in deaerating 3 cc of distilled water, expelling only 1 cc, and treating the 2 cc left in the chamber as if it were a sample to be analyzed.  $C_w$  = the correction for change in water vapor corresponding to any temperature change between the blank and the sample for analysis.  $C_w = (t_a - t_0) \Delta p_w$  where  $t_a$  = the temperature of the sample analysis and  $t_0$  = the temperature of the blank analysis.  $\Delta p_w$  = the change in water vapor tension corresponding to 1° of temperature change in the temperature region of the analysis.  $f_{\text{N}_2\text{O}}$  = the manometric factor for  $\text{N}_2\text{O}$  calculated from a factor  $z$  of 1.03 (Orcutt and Waters (10)) and values of 0.507 and 0.438 for the  $\alpha'$  of the analysis mixture at 20° and 30° respectively.

The Bunsen solubility coefficient ( $\alpha$ ) is calculated from the  $\text{N}_2\text{O}$  concentration in volume per cent in the blood or homogenate as follows

$$\alpha = \frac{\text{N}_2\text{O vol } \%}{100} \times \frac{760}{B - T_w}$$

where  $B$  = the barometric pressure and  $T_w$  = water vapor tension at 37°

In the case of the brain homogenate the  $\alpha$  obtained above is for the brain-water mixture as a whole, but since the  $\alpha$  for water can be determined, and the relative quantities of brain and water are known, the solubility of nitrous oxide per gm of brain can readily be calculated

$$\alpha_b = \frac{\alpha_h \left( \frac{W_b}{1.05} + V_w \right) - V_w \alpha_w}{W_b}$$

where  $\alpha_b$ ,  $\alpha_h$ ,  $\alpha_w$  = the Bunsen coefficients for 1 gm of brain, 1 cc of

homogenate, 1 cc of distilled water respectively, all at 37°  $W_b$  = the weight of the brain sample,  $V_w$  = the volume of water, and 1.05 = the specific gravity of brain

*Determination of Brain and Cerebral Venous Nitrous Oxide Concentrations in Vivo*—These studies were performed on dogs anesthetized with sodium pentobarbital. A T-tube, attached to inspiratory and expiratory flutter valves through which gas mixtures could be administered without rebreathing, was introduced into the trachea. The skull was trephined through the occipital protuberance and a threaded brass cannula was screwed into the skull, tapping the torcular Herophili (confluence of the sinuses). A solution of heparin was used in this cannula to prevent clotting. A preliminary period of inhalation of 100 per cent oxygen for 1 hour insured practically complete denitrogenation of the brain. The animal was then permitted to breathe a mixture of 40 per cent nitrous oxide and 60 per cent oxygen. At a variable time after the beginning of nitrous oxide inhalation a sample of cerebral venous blood was collected anaerobically from the cannula in the torcular and immediately thereafter the animal was sacrificed by passage of an electric current through the thorax, a procedure which produced instantaneous ventricular fibrillation. The scalp and muscles were quickly removed from the cranium and two holes 1 inch in diameter were made by means of a trephine on opposite sides of the skull just above the zygomatic arch, with care not to cut completely through the bone. The head was then removed and immersed in a bath of water through which a fine spray of 40 per cent nitrous oxide-60 per cent oxygen had been passing for about 30 minutes. Under the surface of this bath, the trephine holes were completed and the buttons of bone removed. The dura was then cut and a sample of brain was taken anaerobically by means of the following technique. It was found possible to perform all of these operations from the sacrifice of the animal to removal of the brain sample in about 10 minutes, the brain being exposed to the water bath for only 30 seconds.

The brain sampler (Fig. 1) is simply a 10 cc. all-glass syringe in which the end of the barrel has been cut off and the rim beveled to a fair cutting edge. This end may be closed by means of a snugly fitting rubber stopper through the center of which passes a stout capillary tube of 2 mm. bore, extending 8 cm. beyond the rubber tube and ending in a beveled and tapered tip. This tube must fit the stopper tightly enough to resist being forced up or down in it. The entire unit fits into a metal holder which secures the stopper against the open end and permits the plunger to be forced down the length of the barrel by means of a screw-thread, forcing a fine cord of brain tissue from the capillary tip. To obtain a sample of brain, the syringe is immersed in the water bath and the plunger worked back and forth until it moves very

freely. It is then pushed down just beyond the cutting edge of the barrel. This is now pressed against the brain and, with a slightly twisting motion, forced through the tissue, leaving the plunger free to move out as it is displaced by brain. When the cutting edge reaches the other side of the brain, it is closed by means of the rubber stopper and capillary tube. At this point pressure is applied against the plunger and some of the brain forced down the capillary to the tip. In this manner it is possible anaerobically

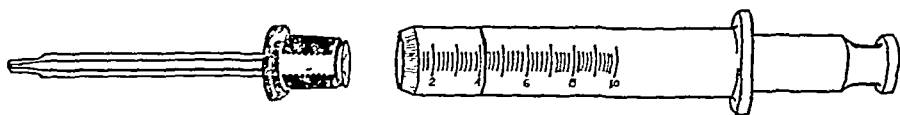


FIG 1 The modified glass syringe used in obtaining samples of brain anaerobically

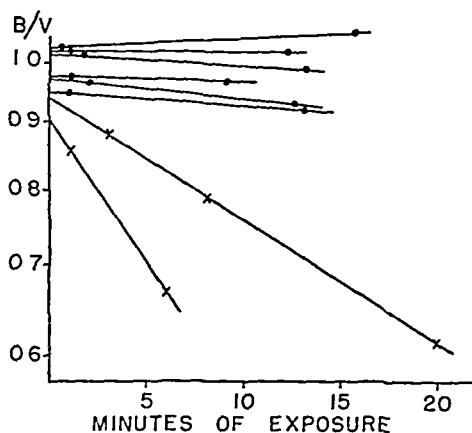


FIG 2 The ratio of the nitrous oxide contents of brain and cerebral venous blood plotted semilogarithmically against the time of exposure of the brain before sampling. X, samples obtained when the bath was equilibrated with 100 per cent O<sub>2</sub>; ●, when equilibration was effected with the N<sub>2</sub>O tension breathed by the animal.

to obtain a satisfactory 6 to 8 cc sample of brain, representing both gray and white matter, sealed within a syringe.

The brain sampler was then removed from the bath, fastened into its holder, and an analysis for nitrous oxide carried out on 2 cc samples by the analytical technique described above for blood and brain homogenate. Samples of torcular blood obtained at the time the animal was sacrificed were also analyzed for nitrous oxide by the same technique. The purpose of the water bath equilibrated with a tension of nitrous oxide equivalent to that inhaled by the animal is to minimize the loss of this gas from the brain in the brief time of exposure as the sample is being taken. This pre-

caution was found to be necessary by preliminary experiments in which simply denitrogenated water was used as the bath (Fig 2) It was found that there was a significant loss of nitrous oxide from the brain with time, if successive samples were taken after the dura was cut When water equilibrated with 40 per cent nitrous oxide was used, however, it may be seen that this loss of nitrous oxide from the brain was prevented It is worthy of note that in the earlier trials, in which loss occurred, if an exponential extrapolation is made to time of exposure = 0 the values obtained are comparable to those in which loss is actually prevented

TABLE I  
*Solubility of Nitrous Oxide in Dog and Human Whole Blood\**

Dog		Man	
Animal No	$\alpha_{N_2O}^{37^\circ}$ (per cc blood)	Red blood cell hematocrit	$\alpha_{N_2O}^{37^\circ}$ (per cc blood)
		<i>per cent</i>	
9	0.419	28.8	0.400
11	0.419	34.2	0.408
12	0.433	41.0	0.410
13	0.421	44.0	0.414
14	0.435	51.5	0.425
15	0.421		
Mean	0.425		0.412
S. E.	0.003		0.004

\*  $\alpha_{N_2O}^{37^\circ}$  (per cc of blood) = the number of cc of  $N_2O$  (reduced to S.T.P.) dissolved by 1 cc of blood when equilibrated at a nitrous oxide tension of 760 mm

### Results

*Solubility of Nitrous Oxide in Blood and Brain in Vitro*—The Bunsen solubility coefficient (Table I) for nitrous oxide in dog blood at  $37^\circ$  as determined by this technique was 0.425 (standard error = 0.003), this coefficient for human blood was 0.412 (standard error = 0.004). In the latter case a definite correlation may be noted between the proportion of red cells in the blood and the solubility of nitrous oxide in it. This was found to be the case for nitrogen by Van Slyke, Dillon, and Margaria (11). We found that the blood of a very anemic and a polycythemic individual varied from the mean by only 3 per cent. The values for  $\alpha$  obtained by us in blood agree well with the value of 0.416 found by Orcutt and SeEVERS (12). The solubility of nitrous oxide in brain, however, has not hitherto been investigated. We found identical values for the whole brain of dog ( $0.437 \pm 0.008$ ) and of man ( $0.437 \pm 0.005$ ), the former studied immediately after sacrifice, the

latter obtained in autopsy specimens 4 to 24 hours after death from a variety of diseases (Table II). From these values for blood and brain in both species a brain-blood partition coefficient of 1.03 and 1.06 may be calcu-

TABLE II  
*Solubility of Nitrous Oxide in Dog and Human Whole Brain\**

Dog		Man		
Animal No	$\alpha_{N_2O}^{37^\circ}$ (per gm brain)	Patient	Condition	$\alpha_{N_2O}^{37^\circ}$ (per gm brain)
8	0.434	A. K.	Depressive psychosis	0.428
9	0.406	B. B.	Nephritis	0.434
11	0.430	S. F.	Apoplexy	0.437
12	0.420	M. Mc	Cardiac failure	0.425
13	0.458	R. C.	Paresis	0.438
14	0.455	C. H.	Arteriosclerosis	0.432
15	0.454	J. J.	Hypertension	0.464
Mean	0.437			0.437
S. E.	0.008			0.005

\*  $\alpha_{N_2O}^{37^\circ}$  (per gm of brain) = the number of cc of  $N_2O$  (reduced to S.T.P.) dissolved by 1 gm of brain when equilibrated at  $37^\circ$  at a nitrous oxide tension of 760 mm.

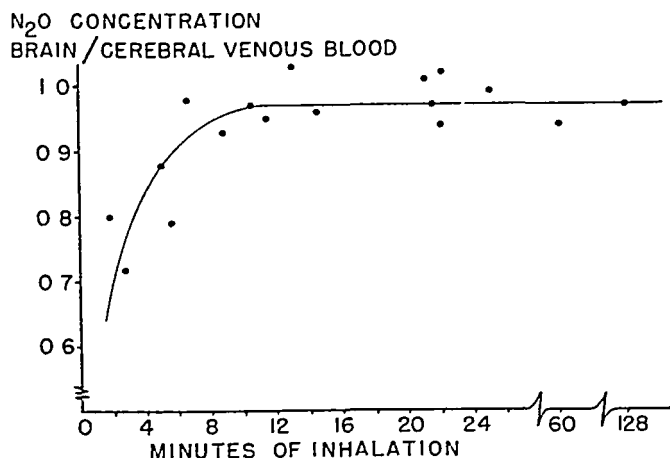


FIG. 3 The rate of equilibration of nitrous oxide between brain and cerebral venous blood *in vivo*.

lated for dog and man respectively, representing the ratio of the quantity of nitrous oxide dissolved in 1 gm of brain to that in 1 cc of blood when both are at the same tension.

*Partition Coefficient in Vivo for Dog and Rate of Equilibration between Brain and Cerebral Venous Blood*—In Fig 3 are presented the data on the nitrous oxide content of brain and cerebral venous blood obtained simultaneously at times varying from 2 minutes to 2 hours after the onset of inhalation of the 40 per cent nitrous oxide mixture. It is seen that in the early period the brain has not come to equilibrium but that after about 10 minutes equilibrium is apparently established between brain and the blood draining the brain and that the ratio of the nitrous oxide content in the two phases remains unaltered up to 2 hours. The value for this brain-blood ratio in the five experiments with equilibration times from 8.6 to 13.3 minutes ( $0.970 \pm 0.008$ ) is not significantly different from that in the six experiments in which equilibration time was 20 minutes to 2 hours ( $0.975 \pm 0.011$ ). It is therefore possible to conclude that equilibration between brain and its venous drainage with respect to nitrous oxide tension is complete within 10 minutes, which is therefore the value of  $u$  in the equation for cerebral blood flow. The value for the brain-blood ratio of nitrous

TABLE III  
*Brain-Blood Partition Coefficient (S) of Nitrous Oxide in Dog and Man*

Technique	Species	S	Standard error
<i>In vitro</i>	Dog	1.03	0.020
" "	Man	1.06	0.016
" <i>in vivo</i> , 8-13 min equilibration	Dog	0.970	0.008
" " 20 min to 2 hrs equilibration	"	0.975	0.011

oxide concentrations at equilibrium (0.975) is the *in vivo* partition coefficient and agrees closely with the value for this constant obtained from *in vitro* equilibration (1.03 for the dog) (Table III).

#### DISCUSSION

An exact value for the solubility of nitrous oxide in the living human brain is of course not directly obtainable, although preliminary experiments in this laboratory have indicated that such values for radioactive gases are capable of direct estimation. Nitrous oxide solubility has, however, been measured in samples of human brain obtained at autopsy. We have demonstrated furthermore that this solubility in human brain tissue is identical with that in dog brain after death which, in turn, is remarkably close to the solubility in the living brain of that animal. It is, therefore, very likely that the same is true for man and that the solubility constant obtained *in vitro* is applicable to the living state. The experimentally determined values for the brain-blood partition coefficient of nitrous oxide

lie on either side of and only slightly removed from unity and it seems fair to accept this value as a best approximation. The identity of the values found in dog and man speak for the dependence of this coefficient only on gross physicochemical constitution, which varies within extremely narrow limits despite major pathological changes (13-15). The small deviations found in the studies on brains from patients dying of a variety of diseases are further evidence for the constancy of this partition coefficient in different patients and diverse pathological states. This value of unity for the partition coefficient is less than the value of 1.3 tentatively accepted on the basis of a few preliminary measurements (7). The numerous refinements in technique herein reported have undoubtedly yielded a more accurate evaluation of this constant.

In view of the high lipide content of brain tissue and the more than 3-fold greater solubility of nitrous oxide in common fats and oils over that in water or blood, it is surprising that the solubility of this gas in brain is not significantly greater than its blood solubility. In fact in two determinations on white matter (which has twice the lipide content of cortex) the nitrous oxide solubility was found to be within the range of that in whole brain (0.427 and 0.468). These findings are, however, not without precedent. In fact the majority of investigators who have studied, by somewhat cruder techniques, the contents in brain and blood of various volatile anesthetics have arrived at similar conclusions (2-6). Especially noteworthy are the studies of Campbell and Hill (16) who found the nitrogen content of the whole brain at the usual atmospheric tensions to be 1.08 volumes per cent, not significantly different from the blood nitrogen content, yet these workers found the solubility of nitrogen in adipose tissue to be 5 times its solubility in blood. The conclusion is inescapable that brain lipides do not behave as do the usual fats and oils or even adipose tissue in their capacity for nitrous oxide and probably other gases. This does not necessarily vitiate the lipide theory of anesthesia, whose proponents may still reasonably ascribe a preferential absorption of volatile anesthetics to certain important lipides in the surface layers of nerve cells, even though the bulk of the lipides of central nervous tissue is indifferent to these gases.

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#### SUMMARY

1. Techniques are described for the determination of the solubility of gases in brain *in vitro* and *in vivo*.
2. The Bunsen coefficient expressed as cc. of nitrous oxide (converted



to standard temperature and pressure) dissolved by 1 gm of brain when equilibrated at a nitrous oxide tension of 760 mm and  $37^{\circ}$  equals  $0.437 \pm 0.008$  and  $0.437 \pm 0.005$  for the mixed brain of dog and man respectively

3 The brain-blood partition coefficient for nitrous oxide at  $37^{\circ}$ , expressed as the solubility per gm of brain divided by the solubility per cc of blood, was found to be 1.03 and 1.06 for dog and man *in vitro*, respectively, and 0.98 for the dog *in vivo*

4 After approximately 10 minutes of inhalation of a constant tension of nitrous oxide there is complete equilibrium in the dog between brain and cerebral venous blood with respect to nitrous oxide tension

5 The pertinence of these determinations to a method for measurement of cerebral blood flow in man and to the lipide theory of anesthesia is discussed

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# MICROBIOLOGICAL EVIDENCE FOR THE IDENTITY OF $\alpha$ - AND $\beta$ -BIOTIN\*

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Kogl (1, 2) has concluded that two forms of biotin exist in nature. The first of these,  $\alpha$ -biotin, was isolated from egg yolk by Kogl and Tonnis (3), and the second,  $\beta$ -biotin, was isolated from liver and milk by du Vigneaud and associates (4-6), and more recently from liver by Kogl and ten Ham (1). Kogl based his conclusion on differences in the physical, chemical, and biological properties of the isolated compounds. There appears to be no doubt about the structure of the  $\beta$ -biotin, as this compound has been synthesized repeatedly and is now an article of commerce. While Kogl proposed a structural formula (7) for the so called  $\alpha$ -biotin, he has not synthesized it. Melville (8), in his review, questions the conclusion that the two isolated biotins are different compounds.

One of the points of difference reported by Kogl was the response of yeast to the two compounds. Kogl and ten Ham (1, 2) reported that the activity of  $\beta$ -biotin was approximately twice that of  $\alpha$ -biotin when tested by the "Rasse M" strain of yeast. Du Vigneaud and coworkers (4, 9) reported that a sample of  $\alpha$ -biotin which they received from Professor Kogl had less than 50 per cent of the activity that their crystalline preparation from liver had for yeast, *Rhizobium trifolii*, and *Clostridium butylicum*. They pointed out, however, that this difference should not be stressed, since the Kogl sample might have suffered loss in potency, or might have been impure. Later revision of the melting point to a higher figure by Kogl and Pons (10) suggests that the earlier samples of  $\alpha$ -biotin were not pure. In view of these differences and since we had a sample of Kogl's biotin, it seemed worth while to compare the activity of the two compounds for a number of microorganisms to see what light such tests might throw on the problem.

## EXPERIMENTAL

*Microbiological Assays*—The organisms used were *Lactobacillus casei*, American Type Culture Collection, No 7469, *L. pentosus* 124-2, *Saccharo-*

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*myces cerevisiae* Y-30, *Clostridium acetobutylicum* S-9, and *Neurospora crassa* 1-A wild

The stock cultures of the lactobacilli were carried, inocula were prepared, and the procedure was carried out as described previously (11). The medium of Shull and Peterson (12) was used for *L. casei*. The medium for *L. pentosus* 124-2 contained, per liter, 10 gm of glucose, 10 gm of sodium acetate, acid-hydrolyzed casein equivalent to 5 gm of casein, 0.1 gm of L-cystine, 0.025 gm of DL-tryptophan, 0.02 gm of adenine sulfate, 0.25 mg of riboflavin, 0.5 mg of calcium pantothenate, 2.5 mg of nicotinic acid, 0.25 mg of p-aminobenzoic acid, and 5 ml each of Salt Solutions A and B (11).

*Saccharomyces cerevisiae* was carried as a slant on the same medium as that for the lactobacilli. The inoculum was prepared by transferring from the slant to 10 ml of the assay medium containing 1 m $\gamma$  of biotin. After 18 to 24 hours growth the culture was centrifuged and the cells were resuspended in 10 ml of sterile water. 1 drop of this suspension was used as inoculum for each flask.

The yeast assays were made in 50 ml Erlenmeyer flasks. The medium contained, per liter, 10 gm of glucose, 6 gm of  $\text{NH}_4\text{H}_2\text{PO}_4$ , 1 gm of  $\text{KH}_2\text{PO}_4$ , 1 gm of asparagine, 1 gm of sodium citrate, 0.25 gm of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 4 mg of thiamine, 4 mg of calcium pantothenate, 4 mg of pyridoxine, 4 mg of nicotinic acid, 100 mg of inositol, and 1.0 ml of the salt solution (0.2 gm of  $\text{MnSO}_4$ , 0.2 gm of  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , and 0.1 gm of  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$  per liter)<sup>1</sup>. The final volume in the flasks was 10 ml. After inoculation the flasks were incubated at 30° on a reciprocating shaker making 90 strokes per minute which had a stroke distance of 10 cm. At the end of 16 hours the flasks were compared by reading light transmission in an 18 × 150 mm tube in an Evelyn photoelectric colorimeter with a 660 m $\mu$  filter.

The inoculum for the *Clostridium acetobutylicum* assays was prepared by transfer from a soil spore stock to a 5 per cent corn mash medium. When this culture was actively gassing, usually after about 24 hours, transfer was made to a synthetic medium described in an earlier paper (13) containing 5 m $\gamma$  of biotin. After 18 hours, another transfer was made to another tube of synthetic medium, this time containing only 1 m $\gamma$  of biotin. This culture was used as an inoculum when it was actively gassing, usually after 12 to 18 hours, 1 drop to each tube.

The assays were conducted as in the method of Lampen and Peterson (13) for the assay of p-aminobenzoic acid, except that 0.1 per cent sodium thioglycolate was employed instead of reduced iron and sodium hydrosulfite. The tubes were read at 18 hours in an Evelyn photoelectric colorimeter with a 660 m $\mu$  filter.

<sup>1</sup> Olson, B. H., unpublished data.

The inoculum for the *Neurospora crassa* assays was that described by Stokes *et al* (14). Approximately 3 sq mm of spores from a slant consisting of 2 per cent agar, 0.2 per cent Difco yeast extract, and 0.2 per cent Difco malt extract, were suspended in 10 ml of sterile water, and 1 drop of this suspension was used as the inoculum.

The assays were run in the medium of Horowitz and Beadle (15) with 10 ml of medium in 50 ml Erlenmeyer flasks. The mycelium was harvested after 5 days incubation at 30°. The pads were pressed out on filter paper, dried 12 hours at 90°, and weighed to the nearest 0.5 mg.

The sample of biotin methyl ester was received from Professor Kōgl as

TABLE I  
Comparative Activity of Kōgl's Biotin and Synthetic *dl*-Biotin

Biotin per 10 ml *	<i>Lactobacillus casei</i> 7469		<i>Lactobacillus pen- tosus</i> 124-2		<i>Saccharomyces cerevisiae</i> Y 30		<i>Clostridium aceto- butylicum</i> S 9		<i>Neurospora crassa</i> 1 A wild	
	Acidity ml 0.1 N NaOH per 10 ml				Evelyn readings, 660 mμ filter				Mg mycelium per 10 ml	
	M †	K †	M	K	M	K	M	K	M	K
0	2.1	2.1	0.8	0.8	96	96	97	97	8	8
0.1	3.6	3.5	2.9	2.8	78	80	86	88	11	10
0.2	4.9	4.8	4.8	4.6	61	65	79	80	18	16.5
0.3					50	50			21.5	20.5
0.4	7.6	7.2	7.8	7.3						
0.5					34	35	70	70	31	29
0.6	10.1	9.9	9.5	9.3						
1.0			10.1	9.8					52	53
Comparative activity	100	90-96	100	94-95	100	90-94	100	90-93	100	90-96

\* The Kōgl sample was corrected for 25 per cent impurity, the amounts of synthetic *dl*-biotin represent only the *d* isomer.

† K, Kōgl sample, M, Merck synthetic *dl*-biotin.

a sterilized solution in 1940, and since that time has been kept in the refrigerator in a sealed ampule at a concentration of 1 γ per ml in 50 per cent methyl alcohol. Since this sample was received before the question of the two forms of biotin had arisen (1, 2), it is presumably the α type. In his letter accompanying the sample, Professor Kōgl stated that the biotin contained about 25 per cent impurity, and hence a 25 per cent correction has been applied in calculation of the amounts used. The methyl ester was hydrolyzed to the free acid before use. The β-biotin used was crystalline synthetic *dl*-biotin from Merck and Company, Inc. It has been shown (16) that only *d*-biotin has microbiological activity.

### Results

Table I shows the comparative activities of the two biotins for five microorganisms. The activity of the Kogl biotin (which had been corrected for the 25 per cent impurity) was consistently from 90 to 96 per cent that of *d*-biotin (no activity for the *l* isomer being assumed). Du Vigneaud and coworkers (4, 9) have likewise reported the same *relative* activity for three microorganisms.

On the basis of these data two alternative assumptions may be made. The first is that the two compounds are identical, and hence have the same chemical and microbiological properties. The second is that the two biotins are chemically different, but elicit the same microbiological response. Some precedent for the latter view is found in the equal activity of biotin and oxybiotin for *Lactobacillus arabinosus* 17-5 (17-19) and *L. pentosus* 124-2<sup>2</sup>. However, oxybiotin is less potent than biotin for several other organisms, *e g* yeast (18-20) and *L. casei* (17-20). It is therefore hardly to be expected that, if the two biotins are not the same compound, they would have identical activities for five microorganisms as diverse as one anaerobic and two lactic acid bacteria, a yeast, and a mold. The microbiological data then lend support to the view that the two biotins are identical rather than different compounds.

### SUMMARY

The activity ratio of Kogl's  $\alpha$ -biotin to synthetic *dl*-biotin ( $\beta$ -biotin) for each of five microorganisms has been determined. In all cases Kogl's  $\alpha$ -biotin was found to possess 90 to 96 per cent of the activity of the synthetic *dl*-biotin, assuming only *d*-biotin is active.

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# THIAMINE AND THE CYANOGEN BROMIDE REACTION\*

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Most chemical methods for the determination of nicotinic acid involve reaction with cyanogen bromide and an aromatic amine (1) or ammonia buffer (2). In some cases (3-10) heat is applied to the nicotinic acid-cyanogen bromide reaction mixture. During the course of investigations involving use of this reaction, it was found that thiamine, under certain conditions, also produces a colored compound with the reagent. The importance of this fact is appreciated when one considers that in general such determinations are carried out with the assumption that nicotinic acid is the only substance present which, with cyanogen bromide, will produce a colored compound measurable at the specific wave-length designated. Obviously, the presence of any other compound possessing this property could affect the accuracy of a nicotinic acid assay. Thiamine apparently is such a substance, since investigation has revealed that this vitamin, if present in relatively high concentration, does produce a colored compound with cyanogen bromide when the reaction is carried out at an elevated temperature.

## EXPERIMENTAL

In studying the effect of thiamine on the cyanogen bromide reaction, the general procedure previously recorded for the determination of nicotinic acid (1) has been followed with respect to sample volume, cyanogen bromide reagent, buffer, and, when used, the amine reagent (*m*-phenylenediamine). In all colorimetric measurements a Klett-Summerson photoelectric colorimeter was employed. Since it was noted that addition of the aromatic amine was not required for the color to be produced by interaction of thiamine and cyanogen bromide, a study was made of the reaction without use of the amine. To accomplish this, 10 ml samples of the thiamine solution plus 5 ml of a buffer of pH 6.6 were heated on a steam plate for 10 minutes with 5 ml of a 4 per cent aqueous solution of cyanogen bromide. Colorimetric readings (440 m $\mu$ ) were made at varying times after removal from the steam plate. Table I presents the results so obtained. From these data it is apparent that the reaction between thiamine and cyanogen

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bromide is slow but that, given sufficient time, it is complete and the color developed is proportional to the amount of thiamine taken. The high concentration of thiamine required, however, makes the reaction impracticable as a general assay procedure for this vitamin.

The production of color by thiamine in the presence of cyanogen bromide is not dependent upon the presence of an aromatic amine. Therefore, any cyanogen bromide-nicotinic acid assay procedure in which heat is employed might be subject to interference by the presence of a high concentration of

TABLE I  
*Determinations with Pure Thiamine Solutions*

Thiamine in sample	Density scale readings at varying times after removal from steam plate					
	15 min	30 min	45 min	1 hr	2 hrs	3 hrs
<i>mg per ml</i>						
0 00	0	0	0	0	0	0
0 05	46	50	52	55	57	57
0 10	70	80	82	90	105	110
0 15	105	120	125	135	158	165
0 20	140	158	166	180	210	220

TABLE II  
*Determination of Nicotinic Acid in Presence of Thiamine*  
- of nicotinic acid in 10 ml of sample

Thiamine added	Density scale reading	Apparent recovery of nicotinic acid	
		$\gamma$	per cent
<i>mg per ml</i>			
0 00	130		
0 00	130		
0 05	150	57.7	115.4
0 10	175	67.3	134.6
0 15	200	76.9	153.8
0 20	222	85.4	170.8

thiamine. Such an effect is seen in Table II. In obtaining these data, the previous procedure (1), including use of *m*-phenylenediamine, was followed with the exception that the reaction mixture was heated (45–50°) for 5 minutes after addition of the cyanogen bromide and then placed at room temperature for 15 minutes before the aromatic amine was added. Heating was limited to 5 minutes, since further heating destroys the color produced by nicotinic acid, and since it is the maximum time employed in most procedures which include this step. The temperature was considerably lower than that used in methods requiring heat and serves to show that

for color production with thiamine the heat treatment does not need to be extreme. It is obvious from the data of Table II that under these conditions a nicotinic acid assay, in the presence of a high thiamine concentration, would be subject to error. Apparently, however, the reaction with thiamine is so slow that no error is involved in determinations in which the cyanogen bromide reaction is carried out at room temperature.

#### SUMMARY

Thiamine, under certain conditions, produces with cyanogen bromide a colored compound measurable at the wave-lengths commonly employed for the quantitative determination of nicotinic acid. Since production of this colored compound results when the thiamine concentration is relatively high and when the cyanogen bromide reaction is carried out at an elevated temperature, a potential analytical hazard exists in those nicotinic acid assay procedures in which heat is applied to the reaction mixture.

Although interference by nicotinic acid can easily be overcome by prolonging the time of heating, utilization of this reaction for the quantitative determination of thiamine does not appear to be generally practicable because of the high concentration of thiamine required.

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# A PHOTOMETRIC METHOD FOR THE DETERMINATION OF FREE PENTOSE IN ANIMAL TISSUES\*

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Most of the micromethods for the determination of pentoses in tissues are based upon the formation of furfural and the reaction of the latter compound with such reagents as aniline (1), benzidine (2), xylidine (3), or orcinol (4). The furfural is formed in these methods usually by heating the unknown in a fairly concentrated solution of strong mineral acid at temperatures ranging from 100–175°. The drastic conditions used in these methods produce furfural not only from pentoses and pentosans but also from other carbohydrates, glucuronic acid (1), and ascorbic acid (5). Methods involving such severe conditions are thus highly non-specific for free pentoses in tissue extracts.

We have developed a method for the determination of free pentose in animal tissues after the administration of pentose. The basis of our procedure is the reaction of *p*-bromoaniline acetate with furfural. The reaction is carried out at 70°, an adaptation that obtains a high degree of specificity.

Acetic acid in a concentration of approximately 83 per cent was found to be more favorable for the conditions of our procedure than strong mineral acids. It was observed that an interfering brown color is produced when an acetic acid solution of aniline mixed with tissue filtrate is heated. This brown color is due to the presence of oxidation products of aniline. A study of aniline derivatives showed that the *p*-bromo-substituted compound is more resistant to the formation of the interfering colored products than is aniline, hence the use of *p*-bromoaniline was adopted.

Another improvement of great importance was the addition of an antioxidant, thiourea, to the color-producing reagent. The reagent finally developed consists of 2 per cent *p*-bromoaniline in glacial acetic acid saturated with thiourea.

The problem of preparing tissue extracts not containing objectionable amounts of interfering substances was solved by using Somogyi's deproteinizing reagents (6), barium hydroxide and zinc sulfate. The Somogyi reagents yield filtrates essentially free from combined pentose compounds from which small amounts of furfural would be formed by heating with our

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color-producing reagent These filtrates, however, contain some interfering substances, such as glucuronic acid and glucose Interference from such substances is avoided by preparing a tissue extract of 1/20 dilution or greater With 1/20 filtrates of muscle, brain, kidney, spleen, blood, and hide of the fasted rabbit, practically no color is obtained by our procedure However, with liver filtrates of a 1/20 dilution a color is obtained which amounts to about 6 mg of apparent pentose per 100 gm of tissue Glycogen in liver filtrates does not yield interfering color but produces turbidity This objection is not serious, as experimental work is usually carried out on fasted animals

TABLE I

*Interference Observed with Authors' Method and with Orcinol Reagent at 70°*

Compound	Color produced by 0.1 mg D-ribose per cc			
	Authors' method, mg per cc		Orcinol reagent at 70°, mg per cc	
	0.1	1.0	0.1	1.0
	per cent	per cent	per cent	per cent
Glucose	1.0	8.5	5.1	8.9
Fructose	0.0	0.0	7.1	127.0*
Ascorbic acid	0.0	0.0	6.5	42.3
Dehydroascorbic acid	0.0	2.1	0.8	34.2
Na glucuronate	0.0	2.4	5.4	53.5
Galactose	4.0	21.6	1.6	5.3
Sucrose	0.5	1.0	3.9	43.5
Maltose	0.0	0.0	6.5	8.3
Lactose	0.0	1.8	2.5	3.3
Soluble starch	0.0	0.0	5.1	5.7
Glycogen	0.0	0.8	27.6	32.2
Gum arabic	5.2	22.2	39.5	114.0

\* Solution became turbid

We found the orcinol reaction of Bial less satisfactory for the determination of pentoses than the reaction with *p*-bromoaniline as developed by us When the orcinol reagent is mixed with test material and boiled at 100° for 5 to 20 minutes, as recommended by different authors (4, 7), the interference from non-pentose material is very great Even under the mild conditions of our procedure the orcinol reagent gives considerable interference from non-pentose substances, as shown in Table I The data of Table I were obtained by mixing 1 cc of unknown solution with 5 cc of reagent, warming in a water bath at 70° for 10 minutes, and cooling promptly to room temperature The orcinol reagent was essentially the same as that used by Mejsbaum (4), Miltzer (7), and others It consisted of a mixture

of 1 volume of 1 per cent aqueous orcinol, 0.1 volume of 10 per cent  $\text{FeCl}_3$  solution, and 4 volumes of concentrated  $\text{HCl}$ , freshly prepared. The data show that greater interference results with the modified orcinol method than with the authors' procedure.

### *Technique of New Method*

#### *Reagents—*

1 *p*-Bromoaniline reagent Thiourea is added to glacial acetic acid in excess of the amount that will dissolve. Approximately 4 gm of thiourea per 100 cc of acetic acid are used. Decant 100 cc of acetic acid saturated with thiourea and dissolve 2 gm of pure *p*-bromoaniline (Eastman Kodak Company) in it. Keep the *p*-bromoaniline reagent in a dark glass bottle and prepare about once weekly.

2 *Somogyi deproteinizing reagents* (6) 5 per cent solution of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 0.3 *N* barium hydroxide solution are prepared. These reagents must neutralize each other precisely, volume for volume, when titration is performed with phenolphthalein as indicator.

3 *Sugar standard* A stock solution of pentose is made up by dissolving reagent grade sugar in saturated benzoic acid solution. A working standard containing 0.1 mg per cc is prepared by diluting the stock solution with saturated benzoic acid.

### *Procedure*

The filtrate of the tissue is made with an expected concentration of 0.01 to 0.2 mg per cc. The following procedure is used for a 1:20 dilution. Place in a Waring blender, or a mortar, a convenient quantity of tissue. Add 15 volumes of water and homogenize until a thoroughly dispersed mixture is obtained. Add 2 volumes of 0.3 *N* barium hydroxide and mix thoroughly. Now add without delay 2 volumes of 5 per cent  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and mix thoroughly. Filter.

Place 1 cc of filtrate in each of two photoelectric colorimeter tubes. In each of two similar tubes place 1 cc of standard pentose solution containing 0.1 mg per cc. To each of the tubes add 5 cc of the *p*-bromoaniline reagent and mix thoroughly. Place one tube containing the standard solution and one tube with the unknown filtrate in a water bath at  $70^\circ$ , keeping a standard and an unknown tube to serve as unheated blanks. Keep the tubes in the water bath for 10 minutes, then remove, and cool in running water until the tubes have reached room temperature. Set the tubes in the dark for 70 minutes, then read in a photoelectric colorimeter, using a  $520\text{ m}\mu$  filter. The unheated tubes are used as blanks for adjusting the colorimeter. Take the readings and calculate the unknown in terms of the pentose standard used.

TABLE II  
*Agreement with Beer's Law*

	Photometric density, $L = 2 - \log G$		
	D-Arabinose	D-Ribose	D Xylose
$\gamma$ per cc			
100	0 3660	0 3980	0 4410
50	0 1821	0 2007	0 2255
25	0 0908	0 1051	0 1142
12 5	0 0452	0 0537	0 0555
6 25	0 0218	0 0252	0 0298

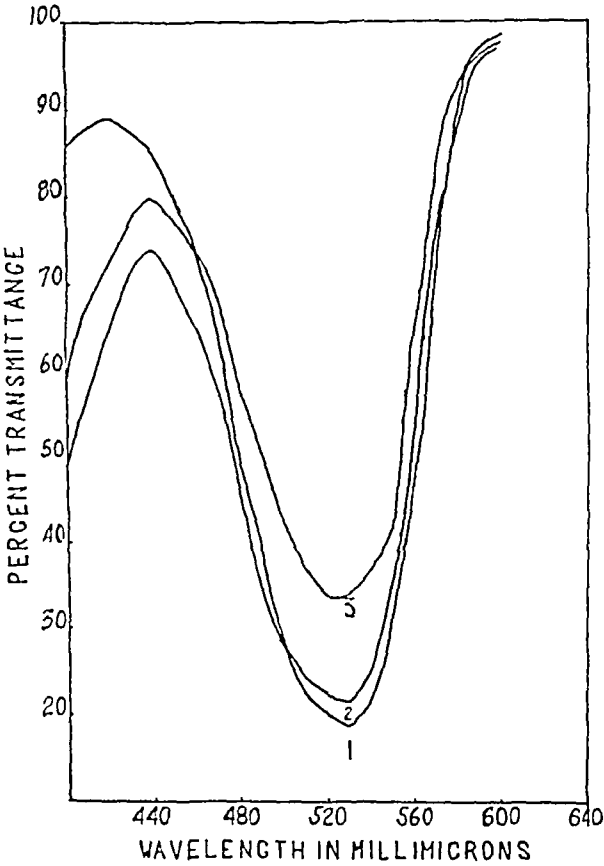


FIG 1 Absorption curves of the chromogen formed by interaction of *p*-bromoaniline reagent with the following Curve 1, furfural, Curve 2, D-xylose, Curve 3, D-arabinose A Coleman spectrophotometer, model 10-S, was used

DISCUSSION

The proportionality of color obtained in this method is in excellent agreement with Beer's law This is shown by the data of Table II for D-arabi-

nose, D-ribose, and D-xylose. Readings were made with the Evelyn photo-electric colorimeter.

The color obtained in this procedure reaches a maximum in approximately 60 minutes and remains constant for about 30 minutes at temperatures of 20-25°, then fades slowly. It is more stable at lower temperatures. We found it to keep constant in intensity for 48 hours in a refrigerator at 8°. This color fades slowly upon exposure to light.

In view of the effect of temperature and light upon the intensity and stability of this color, we recommend that a standard solution of pentose be used for color comparison instead of attempting to standardize conditions so that calibration curves may be made. The use of a single standard is

TABLE III  
*Experiments Showing Recovery of Pentoses Added to Animal Tissues*

Tissue	Sugar	Amount added	Recovery
		mg per gm	per cent
Liver	D-Arabinose	1.0	93.6
Kidney	"	1.0	98.2
"	"	0.02	96.0
Muscle	"	0.02	101.5
"	D-Xylose	1.0	99.3
Brain	D-Arabinose	2.0	101.8
"	D-Xylose	0.02	94.5
Blood	D-Arabinose	1.0	98.5
"	"	0.02	106.5
Intestine and contents	"	1.0	101.8
" " "	"	0.02	101.3
Urine	"	0.02	103.8

satisfactory because of the excellent agreement of the color intensity with Beer's law.

The absorption curves of Fig. 1 offer good proof that the color obtained in the procedure reported is due to the formation of furfural and the reaction of the latter with *p*-bromoaniline acetate.

The recoveries obtained when pentoses were added to animal tissues are shown in Table III.

In this procedure only about 9 per cent of the available furfural appears to be liberated in 10 minutes at 70°. Approximately 4 times as intense a color is produced by heating at 100° for 5 minutes. One may therefore make this procedure more sensitive by heating the reaction mixture to a higher temperature. This is inadvisable, however, except in analyses of materials free from other furfural precursors.

This method has been successfully used in studies of the metabolic fate



of D-arabinose, L-arabinose, and D-xylose in the rabbit which will be reported later

#### SUMMARY

A method for the determination of free pentoses in animal tissues has been developed. The method is based upon the formation of furfural from pentose in 83 per cent acetic acid containing thiourea at 70° and the reaction of the furfural with *p*-bromoaniline acetate to form a pink-colored product.

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# A STUDY OF THE CONVERSION OF ISOTOPIC NICOTINIC ACID TO N<sup>1</sup>-METHYLNICOTINAMIDE

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On the basis of fluorometric estimations, N<sup>1</sup>-methylnicotinamide chloride (NMN) has been reported to be one of the principal urinary excretion products of nicotinic acid in man (1-5), dogs (6), pigs (7), rats (8, 9), and cotton-rats (10). Huff and Perlzweig (2) have isolated this substance from urine after feeding large doses of nicotinamide to man.

In addition it has been reported that the ingestion of tryptophan results in an increased excretion of NMN (9, 11-13), indicating that this compound may have at least two dietary precursors.

As a preliminary to other experiments dealing with the conversion of tryptophan to NMN, it was necessary to prove the conversion of nicotinic acid to NMN. This has been done by using an isotope tracer technique.

Our results indicate that nearly all of the urinary NMN comes from the ingested nicotinic acid under the conditions employed.

## EXPERIMENTAL

*Synthesis of Labeled Nicotinic Acid*—Nicotinic acid<sup>1</sup> containing C<sup>13</sup> in the carboxyl position was synthesized<sup>2</sup> according to the method of Murray, Foreman, and Langham (14). The final product melted at 227.5-229° (uncorrected) and contained 0.81 per cent excess C<sup>13</sup>, corresponding to an excess of 4.86 per cent C<sup>13</sup> in the carboxyl group. Commercial nicotinic acid (Merck) was used as the control material.

*Feeding of Labeled Nicotinic Acid*—Eight male rats, weighing an average of 339 gm. each, had been maintained for several months on a nicotinic acid-free diet consisting of casein 12, L-cystine 0.15, sucrose 81, cottonseed oil 3, salts 4 (15), and the usual vitamins (16). On this diet rats excrete a negligible amount of NMN (16).

1 gm. of the labeled nicotinic acid was added to 1 kilo of the above ration and fed until completely consumed. Urines were collected daily under toluene and kept at 4° during the 14 day collection period.

*Other Methods*—Estimations of NMN were made by the method of Huff

<sup>1</sup> The C<sup>13</sup> was donated through the courtesy of Dr. Sidney Weinhouse, Houdry Process Corporation, Marcus Hook, Pennsylvania.

<sup>2</sup> Kindly synthesized for us by Dr. Wright Langham, Los Alamos Scientific Laboratory, Santa Fe, New Mexico.

and Perlzweig (17) Spectrophotometric determinations<sup>3</sup> were made on a Beckman spectrophotometer All isotope determinations were made on the Consolidated mass spectrometer of the National Bureau of Standards<sup>4</sup> Samples were prepared for analysis by chromic-sulfuric acid oxidation NMN was extracted and purified from the urine according to the method of Huff and Perlzweig (2) with certain modifications as indicated

### *Results*

5450 ml of urine (including funnel washings) contained 681 mg of NMN as determined by fluorimetric assay, equivalent to a 48.6 per cent return of the ingested nicotinic acid

The NMN was extracted as recommended by Huff and Perlzweig except that absolute methanol was used in place of 95 per cent ethanol, and 25 per cent sodium chloride was used for the permutit elutions After the eluate had been freed of sodium chloride, assay indicated that it contained 286 mg of NMN The picrate was formed in 95 per cent ethanol and allowed to crystallize in the cold After removal of the free picric acid the crystals were separated into two portions by fractional crystallization from 95 per cent ethanol One fraction was only sparingly soluble and consisted of small irregularly shaped crystals which became suspended very readily in the alcohol These crystals melted at 227° (uncorrected) and have not been completely identified The second fraction dissolved more readily in boiling alcohol and crystallized in flat yellow leaflets, or when crystallized from more dilute solutions in short prismatic bars The melting point after repeated recrystallizations was 189–189.5° (uncorrected) and showed no depression when mixed with NMN picrate prepared from synthetic NMN,<sup>5</sup> the melting point of which was 189.5° (uncorrected) The melting point of the picrate of nicotinic acid (recrystallized one time) was 217° (uncorrected), indicating that the isolated picrate contained little, if any, nicotinic acid

When rats, diet, and extraction procedures were employed as described above, NMN picrate was isolated after feeding normal nicotinic acid for comparison with the tagged samples and to guard against any natural isotope shift A picrate having the same crystalline structure and melting point as the tagged compound was obtained

Aliquots of the various purified picrate samples were then treated to obtain the free NMN The procedure used by Huff and Perlzweig (2) was followed except that ether saturated with water was used instead of absolute ether to minimize loss in the water phase When crystallized from a

<sup>3</sup> We wish to thank Dr. George A. Hottel for performing these determinations

<sup>4</sup> We wish to thank Dr. Fred Mohler and his staff for doing these analyses for us

<sup>5</sup> W. A. Taylor and Company, Inc., Baltimore, Maryland

minimum amount of boiling absolute ethanol, typical rosettes were obtained. When crystallized from more dilute solutions, the crystals appeared as prismatic bars.

The free compounds were examined spectrophotometrically, all showing identical absorption peaks at 2650 Å with no evidence of a maximum at 2600 Å characteristic of nicotinamide. Further, when the compounds were compared with one another and to synthetic NMN as a standard, each sample appeared to be 100 per cent pure within the limits of error of weighing the 2 mg samples used. Fluorometric assays of the various samples also indicated approximately 100 per cent purity as compared to synthetic NMN.

Melting points and isotope analyses are indicated in Table I. It is evi-

TABLE I

*Isotope Analyses and Melting Points of Free Compounds Obtained from Picrates*

N <sup>14</sup> -Methylnicotinamide	M p (uncorrected)	Tempera- ture of decom- position	Excess C <sup>13</sup>
	C	°C	per cent
Tagged	230-230.5†	233	+0.664 ± 0.005
Normal	233	233.5	+0.013 ± 0.003
Synthetic‡	230-230.5	233	-0.002 ± 0.003

\* CO<sub>2</sub> from dry ice served as the standard for all analyses. The figures given are the average of at least twelve repeated measurements, the plus-minus figures representing the average deviation from the mean.

† Huff and Perlzweig (2) give 233-234° (uncorrected) as the melting point of NMN.

‡ This corresponds to 4.64 per cent excess C<sup>13</sup> in the carboxyl group as compared to the original 4.86 per cent excess C<sup>13</sup>.

§ Obtained from the picrate of commercial NMN with the same procedure as was used with the tagged and normal samples.

dent from the isotope analyses that the concentration of C<sup>13</sup> in the NMN isolated from urine was almost as great as in the nicotinic acid fed. When allowance was made for the fact that NMN contains 1 more carbon atom than does nicotinic acid, the tagged NMN had  $95.7 \pm 0.7$  per cent of the original C<sup>13</sup> concentration, a dilution of only 4.3 per cent.

No estimates of total yield from the extraction procedures were made, since only the purest portions of the picrate fractions were subsequently converted to the free NMN.

#### DISCUSSION

It seems clear from the data reported that nicotinic acid is a precursor for urinary NMN. Since only 4.3 per cent of the NMN was not tagged,

it is evident that very little came from sources other than the ingested nicotinic acid under the conditions of this experiment

The 4.3 per cent normal NMN found could have come from two sources (1) the nicotinic acid and related compounds present in the rats at the start of the feeding period and (2) NMN synthesized from tryptophan or other sources during the feeding period

Since the whole carcass content of nicotinic acid has been determined in rats (18), it was estimated that these rats contained a maximum of 89 mg of nicotinic acid<sup>6</sup> and related compounds. Assuming that the body stores of nicotinic acid were completely replaced by the ingested nicotinic acid, and assuming further that the same percentage of this nicotinic acid was recovered in the form of NMN as the observed percentage of recovery from the ingested nicotinic acid (48 per cent), one would expect 59.8 mg of the 681 mg of NMN (8.8 per cent) originally present in the urine to be untagged. This is more than sufficient to account for the observed dilution (4.3 per cent).

Hence it seems likely that there was no appreciable synthesis of nicotinic acid from tryptophan or other sources in this experiment. However, since the level of nicotinic acid fed was at least 50 times the physiologically required amount on this diet (16), it might be expected that nicotinic acid synthesis from sources such as tryptophan would have been inhibited perhaps following the law of mass action.

These data have not been interpreted to indicate that rats cannot synthesize nicotinic acid from tryptophan. There is abundant evidence on a non-isotope basis that they can do so under proper circumstances (9, 11, 16).

#### SUMMARY

Rats fed large amounts of carboxyl-labeled nicotinic acid excreted N<sup>1</sup>-methylnicotinamide chloride which contained 95.7 per cent of the original concentration of C<sup>14</sup>, indicating that nicotinic acid is a precursor for N<sup>1</sup>-methylnicotinamide.

Under the conditions of this experiment, rats excreted little, if any, N<sup>1</sup>-methylnicotinamide from sources other than the ingested nicotinic acid and the body stores.

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<sup>6</sup> Actually these rats probably contained only about two-thirds of this amount, since the diet used produces a mild nicotinic acid deficiency. Repeated assays of muscle and liver from rats on this diet have shown about two thirds of the "normal" amount of nicotinic acid. Whole carcass determinations have not been made however.

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# INHIBITION OF COUPLED PHOSPHORYLATION IN BRAIN HOMOGENATES BY FERROUS SULFATE\*

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Previous studies have shown a marked effect of purified preparations of certain neurotropic viruses and of iron salts on glycolysis of mouse brain homogenates. Colorimetric determinations of the iron content of purified and well dialyzed preparations of the Theiler FA strain of mouse encephalomyelitis virus gave values for iron corresponding to the inhibitory action of these virus preparations on glycolysis (1).

Experiments with intermediary metabolites of glucose breakdown pointed to an impairment of the phosphorylating steps leading to the formation of fructose-1,6-diphosphate. The addition of this latter compound to brain homogenate inactivated by ferrous sulfate resulted in a rapid rate of lactic acid production. However, it was not possible to restore glycolytic activity to the inactivated homogenate by adding an excess of the enzymes hexokinase and phosphofructokinase which are known to catalyze the formation of fructose-1,6-diphosphate. A fraction prepared from rabbit muscle ("restoring factor") which had no hexokinase or phosphofructokinase activity fully restored the activity to the iron-inactivated homogenate as well as to homogenates of mouse brains infected with the Theiler FA strain of mouse encephalomyelitis virus.

It is the purpose of this paper to report evidence which indicates that the restoring factor is identical with glyceraldehyde phosphate dehydrogenase (triose phosphate dehydrogenase). The effect of the partial inactivation of this enzyme in the glycolyzing brain homogenates by iron salts is most apparent in the phosphorylation reactions because the energy-rich phosphate which is derived from the oxidation of 3-phosphoglyceraldehyde becomes the limiting factor in the formation of fructose-1,6-diphosphate. It will be shown that some inactivation of phosphofructokinase also occurs in the presence of iron salts after prolonged incubation at 37°.

Finally, a study was made of the factors in brain homogenates which, in the presence of iron salts, inactivate these two glycolytic enzymes.

## *Methods*

The methods used for the preparation of brain homogenate and of co-enzymes were described previously (1). Phosphocreatine was synthesized

\* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.



as the calcium salt (2) and converted into the potassium salt before use. Glucose determinations were made according to the method of Nelson (3). Triose phosphate dehydrogenase was determined by the spectrophotometric test described by Warburg and Christian (4).

### Results

*Effect of Fluoride and Arsenate on Inhibition Produced by Ferrous Sulfate*—Instead of determining glycolytic activity by lactic acid production

TABLE I

*Effect of Potassium Arsenate, Potassium Fluoride, and DPN on Glucose Phosphorylation in Normal and Iron-Inactivated Brain Homogenates*

0.3 ml of brain homogenate in 0.01 M potassium phosphate, pH 7.4, 0.4 ml of 0.03 M glucose and 0.1 ml of a solution containing 50  $\gamma$  of ferrous sulfate  $7H_2O$  per ml (0.1 ml of  $H_2O$  in the controls) incubated for 20 minutes at  $38^\circ$ , then were added 0.1 ml of 0.16 M  $KHCO_3$ , 0.1 ml of 0.1 M ammonium phosphate, pH 7.6, 0.1 ml of 0.15 M potassium arsenate,\* 0.1 ml of 0.07 M  $MgCl_2$ , 0.1 ml of 0.01 M ATP, 0.5 ml of 1 per cent phosphocreatine, 0.1 ml of 4 per cent nicotinic acid amide (NAA),\* 0.1 ml of 0.7 per cent DPN,\* and 0.1 ml of 0.6 M potassium fluoride,\* final volume used 2 ml. Incubation, 30 minutes at  $38^\circ$ . The determinations of glucose were made on the  $Ba(OH)_2$  and  $ZnSO_4$  filtrate.

Additions	Glucose disappearance after 30 min		
	Normal brain homogenates	Iron inactivated brain homogenates	Inhibition
	micromoles	micromoles	per cent
None	3.6	2.9	19†
KF	4.8	4.3	10†
NAA + DPN + KF	7.3	4.3	41
“ + “	8.0	3.8	53
Arsenate + NAA + DPN + KF	4.0	3.9	3

\* Added only when indicated.

† Small amounts of DPN were present in the freshly prepared brain homogenate.

as was usually done in previous studies, glucose disappearance was followed by chemical determination of glucose after deproteinization by  $Ba(OH)_2$ - $ZnSO_4$  (3). Thus it was possible to analyze effects of inhibitors with known modes of action. Potassium fluoride was used to inhibit enolase and potassium arsenate was added to eliminate the energy-rich phosphate produced by the oxidation of phosphoglyceraldehyde (4). Despite addition of an excess of phosphocreatine, optimal phosphorylation of glucose was not obtained in the absence of DPN (diphosphopyridine nucleotide). Moreover, ferrous sulfate had little inhibitory effect under these conditions (Table I). When DPN was added, glucose phosphorylation was doubled in the normal brain homogenate but was without effect in the iron-inacti-

vated brain. This lack of DPN effect in the inactivated brain was observed, both in the presence and absence of potassium fluoride. Since potassium fluoride blocks the formation of phosphopyruvic acid from phosphoglyceric acid, the only energy-rich phosphate which can be obtained under such experimental conditions is derived from the oxidation of phosphoglyceraldehyde to phosphoglyceric acid. This reaction is catalyzed by the phosphoglyceraldehyde dehydrogenase (oxidizing enzyme) and requires the presence of DPN and inorganic phosphate.

Upon addition of arsenate which can replace inorganic phosphate in this reaction, the production of energy-rich phosphate is avoided, without interference with the oxidation of phosphoglyceraldehyde. It can be seen from

TABLE II

*Stimulation of Glucose Phosphorylation in Iron-Inactivated Brain Homogenates by Fructose-1,6-diphosphate*

Brain homogenate and solutions as in Table I. No addition of phosphocreatine, potassium fluoride, or arsenate. Preliminary incubation with ferrous sulfate and glucose for 20 minutes at 38°, then for 60 minutes at 38° after addition of MgCl<sub>2</sub>, ATP, DPN, NAA, and KHCO<sub>3</sub>.

Brain preparation	Addition	Glucose disappearance <i>micromoles</i>
Normal mouse brain homogenate		13.8
Same + 5 $\gamma$ ferrous sulfate		2.1
“ + 5 “ “ “	Fructose-1,6 diphosphate, 2 micromoles	6.4

Table I that in the presence of potassium arsenate and potassium fluoride iron salts have no inhibitory effect even when DPN is present.

These experiments strongly point, therefore, to the glyceraldehyde phosphate dehydrogenase as the locus of inhibition by iron salts.

*Restoration of Glucose Phosphorylation to Iron-Inactivated Brain Homogenates*—Inhibition of the triose phosphate-oxidizing enzyme was, however, in apparent contradiction to previously obtained results showing rapid lactic acid production from fructose-1,6-diphosphate in brains infected with the Theiler FA virus or inactivated by iron salts.

The large amounts of fructose-1,6-diphosphate which were added in these earlier experiments cannot be compared to the small quantities present in the course of glucose phosphorylation. It was necessary, therefore, to test the effect of small amounts of fructose-1,6-diphosphate on the disappearance of glucose. It was found (Table II) that 2 micromoles of fructose-1,6-diphosphate caused the disappearance of 4 to 5 micromoles of glucose in the homogenate, in addition to the 2 micromoles which are phos-

phorylated in the absence of fructose-1,6-diphosphate. This effect of fructose-1,6-diphosphate on glucose phosphorylation confirmed on the one hand the utilization of this substance in the inactivated brain and stressed on the other hand the impairment of the phosphorylation mechanism in the presence of iron salts.

These three observations, namely the utilization of fructose-1,6-diphosphate, the impairment in the activity of the triose phosphate-oxidizing enzyme, and the capacity of hexose diphosphate to stimulate glucose phosphorylation in the inactivated brain homogenate, led to the following working hypothesis of the mechanism of the iron salt or virus inhibition.

Production of energy-rich phosphate is the limiting reaction in brain homogenates. A partial inactivation of the coupled phosphorylation by the triose phosphate-oxidizing enzyme leads to a marked depression of the energy-rich phosphate supply which, in turn, results in impaired production of hexose diphosphate. If the oxidation of glyceraldehyde phosphate is not the limiting factor, such a vicious circle could block effectively phosphorylation of glucose without noticeably impairing lactic acid production from fructose-1,6-diphosphate.

To test this hypothesis the following experimental approach was used: (a) The triose phosphate-oxidizing enzyme was prepared from yeast and from rabbit muscle, and during the course of purification parallel determinations of enzyme activity and measurements of restoring capacity were made. (b) The final product of purification from rabbit muscle which was recrystallized several times was tested for restoring capacity and for inactivation by the brain homogenates in the presence of iron. (c) Attempts were made to restore to a large extent glucose phosphorylation by a supply of energy-rich phosphate from a source other than oxidation of glyceraldehyde phosphate.

*Purification of Glyceraldehyde Phosphate Dehydrogenase from Yeast and Muscle*—Purification of the phosphoglyceraldehyde-oxidizing enzyme of yeast was carried out according to the description by Warburg and Christian (4). All the fractions of the purification procedure were tested for glyceraldehyde phosphate dehydrogenase activity spectrophotometrically, and for presence of restoring factor by adding them to brain homogenates inactivated by ferrous sulfate.

The details of the purification procedure need not be repeated. The following observations were made with the use of Fleischmann's (bakers') yeast which was dried in thin layers for 5 days at room temperature.

Approximately 60 per cent of the total activity of the phosphoglyceraldehyde dehydrogenase is precipitated with 35 volumes per cent of acetone. By further addition of acetone to 55 volumes per cent, one-fourth of the

total activity can be recovered For further purifications only the first acetone precipitate was used

The isoelectric precipitates were all collected and tested because of observations made by Meyerhof and Junowicz-Kocholaty (5) with American bakers' yeast that a departure from the original method was required In our experience with Fleischmann's (bakers') yeast, the purification procedure of Warburg and Christian (4) can be followed without modification Most of the activity remained in the supernatant at pH 4.5 and was precipitated

TABLE III

*Comparison of Glyceraldehyde Phosphate Dehydrogenase and Restoring Activity in Fractions from Bakers' Yeast Maceration Juice*

Determination of glyceraldehyde phosphate dehydrogenase activity in the Beckman spectrophotometer (4) Units are expressed as the change in density for the 1st minute multiplied by 1000 1 unit of restoring activity is expressed as the capacity to bring about 50 per cent restoration to an iron-inactivated brain homogenate

	Glyceraldehyde phosphate dehydrogenase		Restoring factor	
	Units per ml	Specific activity	Units per ml	Specific activity
1st extract	150,000	2,400	900	14
35% acetone ppt	260,000	6,000	600	15
50% " "	112,000	4,300	350	13
pH 4.5 ppt	200,000	8,000		
" 4.5 supernatant	42,000	5,000	120	14
Nucleic acid ppt	560,000	8,600	1500	23
" " supernatant	13,000	4,300	30	10
After heating*	260,000	30,000	450	54

\* The heating procedure was applied to the nucleic acid precipitate after removal of the nucleic acid by protamine sulfate

upon addition of nucleic acid The nucleic acid was removed with protamine sulfate (Squibb)

The restoring activity of the yeast fractions was measured by determining the amount required to give 50 per cent restoration to a brain homogenate inactivated under standardized conditions A linear relationship between per cent restoration and amounts added was established only for a narrow range in the neighborhood of 50 per cent restoration The activity determination lacked accuracy unless measured in this range From Table III it is apparent that fairly good agreement exists between the phosphoglyceraldehyde dehydrogenase activity and the capacity to restore glycolysis to

the iron-inactivated brain homogenate in all the fractions except for the first crude extract, which showed a relatively higher restoring capacity

Phosphoglyceraldehyde dehydrogenase has been crystallized from rabbit muscle by Cori *et al* (6) by a simple and reproducible method. The product obtained by this method was recrystallized six times and tested for restoring activity after it had been well dialyzed and then diluted in the presence of cysteine. It was found to be very active in restoring glycolytic activity to the iron-inactivated brain.

During the purification procedure a comparative study of the relative triose phosphate dehydrogenase and restoring activity in fractions obtained from rabbit muscle was not made because of the following two difficulties encountered with the muscle preparations: (a) The spectrophotometric determination of the triose phosphate-oxidizing enzyme is not feasible with the crude extracts because of the presence of large quantities of  $\alpha$ -glycerophosphate dehydrogenase which interfere with activity measurements. (b) A concentration of 0.02 M ammonium sulfate inhibits glycolysis almost completely, indicating a high sensitivity of the brain homogenate to this salt. This difficulty was not fully appreciated in earlier experiments.

*Effect of Brain Homogenate and Iron Salts on Added Purified Triose Phosphate-Oxidizing Enzyme*—When a preparation of a six times recrystallized glyceraldehyde phosphate dehydrogenase from rabbit muscle is added to a brain homogenate, it is rapidly inactivated if iron salts are present. Some inactivation, although at a slower rate, occurs in the absence of iron salts. Large quantities of the triose phosphate enzyme had to be added to allow for subsequent dilution in order to avoid interference by the brain homogenate in the spectrophotometric test. In a typical experiment 0.1 ml of 0.21 M glucose, 5  $\gamma$  of ferrous sulfate, and a six times recrystallized preparation of glyceraldehyde phosphate dehydrogenase from rabbit muscle were added to 0.3 ml of a freshly prepared brain homogenate and the mixture incubated at 38° for 15 minutes. For the spectrophotometric test, 0.05 ml of a 1:100 dilution of the mixture in a cysteine-pyrophosphate buffer of pH 8.0 was used. The activity of the diluted sample at zero time was 1000 units per ml (with an experimental error of about 10 per cent). After 45 minutes the 1:100 dilution of the iron-inactivated brain homogenate contained only 300 to 500 units, representing 50 to 70 per cent inactivation, while the activity of the mixture containing normal brain homogenate was depressed by 20 to 30 per cent. These findings are in good agreement with the observations on the over-all glycolytic activity which also falls off slowly in the absence of added ferrous sulfate when subjected to prolonged incubation at 38°.

*Restoration of Glycolytic Activity to Iron-Inactivated Brain by Supply of Energy-Rich Phosphate*—To prove convincingly that in the inactivated

brain homogenate glyceraldehyde phosphate is limiting solely because of its role in the synthesis of adenosine triphosphate (ATP), it was necessary to show that ATP from another source could restore glycolytic activity equally well. Addition of ATP at short intervals has already been shown to have no restoring effect (1). This was not considered conclusive because, as more ATP was added to the brain homogenates, more was destroyed by the active ATPase (adenosine triphosphatase) present.

It was therefore necessary to provide ATP by a continuous reaction from a store of energy-rich phosphate. This was accomplished by the use of

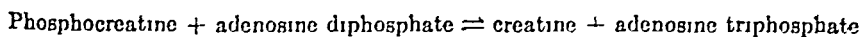
TABLE IV

*Restoration of Glucose Phosphorylation in Iron-Inactivated Brain Homogenates by Addition of Phosphocreatine Enzyme, Phosphocreatine, and Potassium Fluoride*

Experimental conditions as in Table I, except that DPN and nicotinic acid amide were added to all experimental tubes, and in the experiments specified 0.1 ml. of 1.2 M potassium fluoride was added.

Additions	Glucose appearance after 30 min		
	Normal brain homogenates	Inactivated brain homogenates	Inhibition
	micromole	micromole	per cent
None	7.1	1.6	77
KF + phosphocreatine + phosphocreatine enzyme	8.2	6.4	22
Phosphocreatine enzyme	6.5	1.6	75

phosphocreatine and an enzyme from rabbit muscle which catalyzes the reaction



To obtain a valid test, it was necessary to purify this enzyme partially in order to free it of ATPase and triose phosphate dehydrogenase activity. A rabbit muscle extract was dialyzed against distilled water for 5 days and then was precipitated with acetone to remove ATPase activity. The dried acetone powder was extracted with cold water and the clear centrifuged solution fractionated with ammoniacal ammonium sulfate. The fraction obtained at 40 per cent saturation was suitable for the metabolic studies, since it contained no or negligible amounts of ATPase and phosphoglyceraldehyde dehydrogenase. In Table IV the effect of the phosphocreatine enzyme on brain homogenate, inactivated by ferrous sulfate, is recorded. Almost complete restoration of the activity was achieved by addition of the

phosphocreatine enzyme, together with phosphocreatine and potassium fluoride which was added to depress ATPase activity. The enzyme without phosphocreatine was without effect, indicating that it was free of triose phosphate dehydrogenase which restores activity in the absence of phosphocreatine.

The high restoring capacity of glyceraldehyde phosphate dehydrogenase from yeast and muscle, the inactivation of this enzyme when added to brain homogenate in the presence of ferrous sulfate, together with the capacity of ATP (when continuously regenerated from phosphocreatine) to restore activity, leave little doubt that an impairment of the coupled phosphorylation of the triose phosphate is the main cause for the inhibitory effect of iron salts on glycolysis of brain homogenate.

*Effect of Ferrous Sulfate on Phosphofructokinase*—The complete restoration of glycolysis by the addition of purified glyceraldehyde phosphate dehydrogenase pointed to a considerable degree of specificity of the inactivating agent. If present in large excess, other glycolytic enzymes could also have been inactivated under these conditions without affecting the over-all rate of glycolysis, providing their activity was not lowered to the level of the pace-maker.

Earlier experiments on the localization of the inhibition in brain homogenates of mice infected with the Theiler FA virus of mouse encephalomyelitis indicated a slight but consistent inhibition of phosphofructokinase. Addition of purified phosphofructokinase, however, consistently failed to restore glycolytic activity after the inhibitory effect of ferrous sulfate had taken place. Doubts as to the effectiveness of these phosphofructokinase preparations purified from rabbit muscle were removed by testing them on brain homogenates inactivated by incubation at pH 6.3 for 10 minutes at 37°. This procedure has been shown to inactivate phosphofructokinase in mouse brain homogenates (7). As is shown in Table V, the glycolytic activity of an acid-inactivated brain homogenate is fully restored upon addition of phosphofructokinase. The "restoring factor" had no effect under these conditions. The effectiveness of phosphofructokinase in the acid-inactivated brain on the one hand and its lack of restoring capacity in the iron-inactivated brain on the other hand stress the difference in the mechanism of inactivation under these two conditions.

Although it was apparent from these results that the slight inactivation of phosphofructokinase observed in iron-inactivated homogenates is not responsible for the inhibition of the over-all glycolysis, the effect of ferrous sulfate on phosphofructokinase was studied further in the hope of shedding more light on the mechanism of the iron salt effect.

The spectrophotometric determination of phosphofructokinase required the addition of a rabbit muscle fraction which contained restoring factor

Since, at the time the experiments were carried out, the nature of the restoring factor was unknown, it was decided to measure the phosphofructokinase activity by a chemical method directly in the brain homogenate. In the presence of potassium cyanide, ATP,  $MgCl_2$ , and an excess of aldolase (which was readily prepared free of restoring factor), the rate of triose phosphate formation was proportional to the amount of phosphofructokinase present. This method followed closely a procedure described by Herbert *et al* (8) for measuring aldolase activity by determination of alkaline labile phosphate formed. The conditions for the test were as follows. The sub-

TABLE V

*Inhibition of Glycolysis in Brain Homogenates by Acid Treatment and by Addition of "Inactivating Factor" in Presence of Ferrous Sulfate and Cysteine*

Brain homogenate was "acid-treated" by adjusting the pH of the homogenate to 6.3 with 0.01 N HCl, followed by incubation in presence of glucose at 37° for 10 minutes and neutralization with 0.01 N KOH. Phosphofructokinase, "restoring factor," and "inactivating factor" were prepared as described previously (1). The inactivating factor was dialyzed for 3 days against 0.1 M potassium cyanide which was then removed by dialysis against 0.08 M KCl or distilled water for 3 days.

Experiment No	Tissue preparation	Lactic acid production	Inhibition
		$\gamma$	per cent
1	Brain homogenate	2300	
2	Acid-treated brain homogenate + restoring factor	100	96
3	As in Experiment 2 + phosphofructokinase	2200	4
4	Brain homogenate + inactivating factor (dialyzed)	1700	26
5	Same as in Experiment 4 + ferrous sulfate (5 $\gamma$ ) and cysteine (600 $\gamma$ )	560	75

strate was fructose-6-phosphate (0.0025 M), to this were added adenosine triphosphate (0.001 M),  $MgCl_2$  (0.0035 M), NaF (0.06 M), KCN (0.06 M), and 0.1 ml of a 2 per cent solution of aldolase recrystallized from rabbit muscle six or seven times (9). The solution of potassium cyanide was made up freshly and was neutralized before use. Brain homogenate suspended in 0.005 M phosphate buffer at pH 7.5 was added in amounts varying from 500  $\gamma$  to 5 mg of dry weight.

The solutions previously warmed at 38° were mixed, brought to a volume of 2 ml, and finally incubated at 38° for 3 minutes. The reaction was stopped by the addition of trichloroacetic acid. Alkaline labile phosphorus was measured in the filtrate by keeping the solution for 20 minutes at room temperature in 1 N NaOH and correcting for the phosphorus determined on the



untreated filtrate This latter control is necessary for each experiment because of differences in the ATPase activity which leads to the appearance of considerable amounts of inorganic phosphorus from the added ATP, even

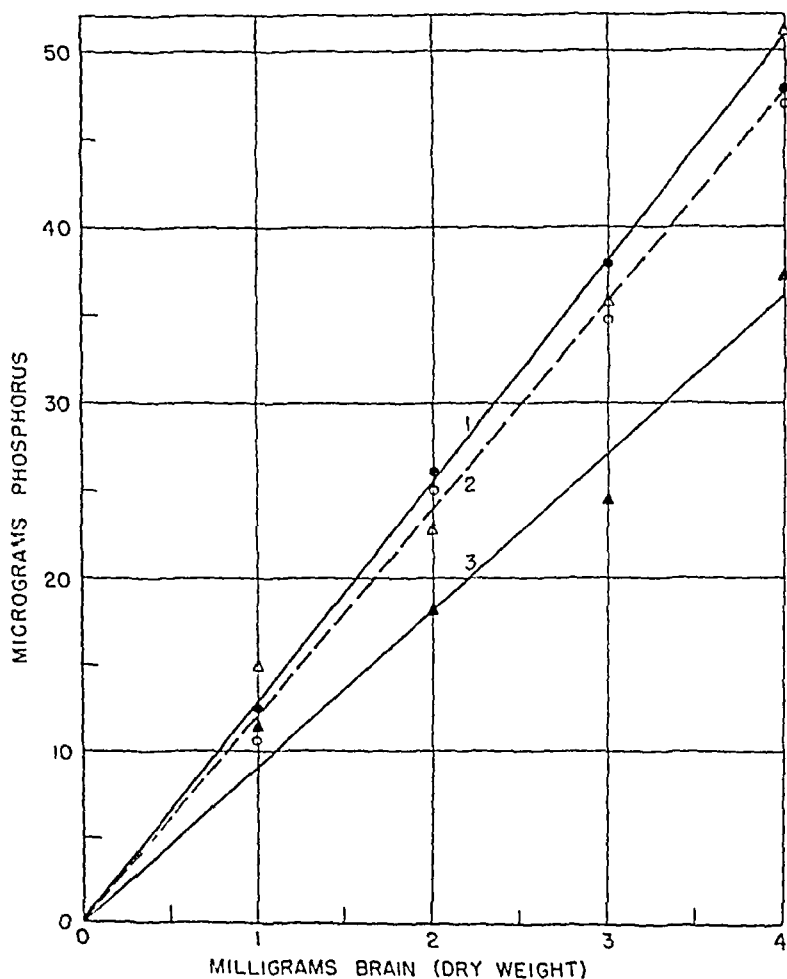


FIG 1 Inhibition of phosphofructokinase activity in brain homogenates by ferrous sulfate Experimental conditions as described in the text Curve 1, phosphofructokinase activity of varying amounts of brain homogenate after incubation for 20 minutes at 38°, Curve 2, ATPase activity of brain homogenate with and without ferrous sulfate, Curve 3, phosphofructokinase activity of brain homogenate incubated for 20 minutes at 38° with 5  $\gamma$  of ferrous sulfate

in the presence of sodium fluoride If corrections are made for the inorganic phosphorus present at the beginning of the experiment, values for the ATPase activity can be obtained from the same data In Fig 1 a typical experiment on the relation between brain concentration and phosphofructokinase activity and the effect of ferrous sulfate on the activity of thi en-

zyme is recorded Also recorded for comparative purposes is the action of ATPase on ATP in presence of sodium fluoride, calculated from the same data While the latter enzyme was not affected by ferrous sulfate, phosphofructokinase showed a consistent though slight inhibition This inhibition could be increased considerably by either prolonging the time of

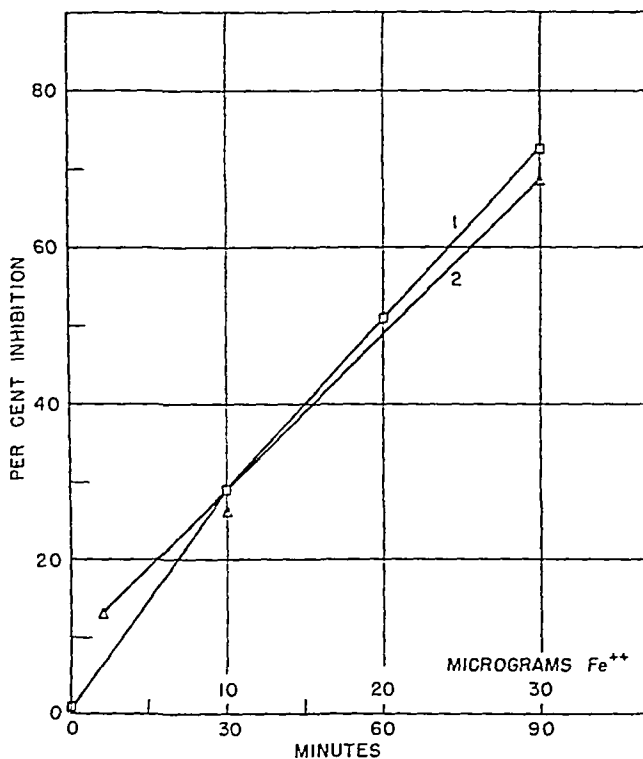


FIG 2 Effect of time and concentration of ferrous sulfate on inhibition of phosphofructokinase activity in brain homogenates Curve 1, effect of time of preliminary incubation in the presence of 5  $\gamma$  of ferrous sulfate on phosphofructokinase activity, Curve 2, effect of varying amounts of ferrous sulfate during preliminary incubation (20 minutes) on phosphofructokinase activity

incubation with ferrous sulfate, or by increasing the ferrous sulfate concentration (Fig 2) It should be noted here that DPN and glucose have a marked protective effect on the triose phosphate dehydrogenase activity but fail to show such an effect on phosphofructokinase

*Mechanism of Ferrous Sulfate Effect*—The dependence of the iron effect on a heat-labile factor, which is present in fresh brain homogenate but which is absent in extracts prepared from acetone-dried brain powder, was previously demonstrated (1) The concentration and purification of this fac-

tor were accomplished by centrifugation of the fresh brain homogenate. The tissue particles thus obtained were washed twice with 0.01 M potassium phosphate buffer at pH 7.5. These preparations inhibited glycolysis so strongly that a direct effect could be provoked without a period of preliminary incubation. The preparations were inactivated by dialysis against 0.1 M potassium cyanide at pH 6.0 for 5 days. Full reactivation was accomplished by addition of ferrous sulfate and cysteine (Table V).

TABLE VI

*Inhibition of Glycolysis of Brain Homogenates by "Inactivating Factor" and by Proteolytic Enzymes*

Crystalline trypsin (8  $\gamma$ ), "inactivating factor," or cathepsin was added before preliminary incubation (20 minutes at 37°), glyceraldehyde phosphate dehydrogenase (GPDase) and 0.1 ml of 0.035 M fructose-1,6-diphosphate (HDP) were added after preliminary incubation. Time of incubation, 60 minutes.

Experiment No	Inhibitor	Addition after preliminary incubation	Lactic acid production $\gamma$	Inhibition per cent
1			2100	
2		GPDase	2000	
3		HDP	2200	
4	Trypsin		1100	48
5	"	GPDase	1800	14
6	"	HDP	1500	28
7	Cathepsin		300	86
8	"	GPDase	1300	38
9	"	HDP	1900	10
10	Inactivating factor		400	81
11	" "	GPDase	1900	10
12	" "	HDP	2300	0

The mechanism by which this non-activated factor destroys the enzymatic activity of the triose phosphate dehydrogenase and phosphofructokinase and possibly of other enzymes is still obscure. The effect of time and temperature on the activity of this factor and its heat lability suggested an enzymatic reaction. Since glyceraldehyde phosphate dehydrogenase, the enzyme which is inactivated by the brain factor, is of protein nature, a proteolytic destruction was considered as a possible mechanism of inactivation. Attempts made to demonstrate proteolysis have so far been unsuccessful. The methods used (formol titration and determination of acid-soluble tyrosine) may not have been sensitive enough or may be unsuitable for this system. Preliminary experiments with synthetic substrates (benzoylargininamide and glycylglycylalanine) were negative or inconclusive.<sup>1</sup>

<sup>1</sup> We wish to thank Dr. M. Levy for the benzoylargininamide, Dr. J. Greenstein for a generous gift of glycylglycylalanine, and Mr. B. Mandel for assisting in these experiments.

It was found, on the other hand, that 50  $\gamma$  of crystalline trypsin, added directly to the glycolyzing brain homogenate, or 10  $\gamma$  of trypsin added during the "preliminary incubation" (1) inhibited glycolysis in a manner similar to that observed with the inactivating factor. The effect of trypsin was also reversed by addition of fructose-1,6-diphosphate or by phosphoglyceraldehyde dehydrogenase.

In view of the possible relation of the inactivating factor prepared from mouse brain to an intracellular proteinase, the effect of a cathepsin on glycolysis was studied. A cathepsin was prepared from beef liver according to the method of Anson (10). The effect of this preparation on glycolysis was identical with that of trypsin or the inactivating factor, namely, the inhibition produced was released by the addition of either fructose-1,6-diphosphate or phosphoglyceraldehyde dehydrogenase (Table VI).

#### DISCUSSION

Studies on the effect of inhibitors on a complex chain of reactions, such as takes place during the breakdown of glucose to lactic acid, meet with two distinct types of difficulties. On the one hand, the rate of the over-all reaction is governed by the slowest of the enzymatic reactions and so the effect of inhibitors on the enzymes present in excess may not be readily noticed. On the other hand, an enzyme which is present in excess, as far as the utilization of an intermediary is concerned, may be limiting with regard to another function such as the production of energy-rich phosphate.

In this study on the effect of iron salts on glycolytic activity, the apparent paradox was observed that inhibition of the phosphoglyceraldehyde dehydrogenase manifested itself most strikingly in the phosphorylation of glucose to fructose-1,6-diphosphate, which reaction occurs at a much earlier stage of glycolysis. When fructose-1,6-diphosphate was added, the inhibition of lactic acid production was counteracted. This complete masking of the actual localization of the inhibition can be explained as follows. For the phosphorylation of glucose, energy-rich phosphate has to be supplied. The partial inhibition of the glyceraldehyde phosphate dehydrogenase with its coupled phosphorylation leads to a decreased formation of ATP. This, in turn, results in an inhibition of glucose phosphorylation and, therefore, less substrate (glyceraldehyde phosphate) for the glyceraldehyde phosphate dehydrogenase is formed. Thus, the production of ATP is further reduced. This vicious circle leads to an amplification of the inhibitory effect on over-all glycolysis which is out of proportion to the effect on the single enzyme.

The production of lactic acid from fructose-1,6-diphosphate is hardly affected, most likely because an enzymatic step, other than the oxidation of glyceraldehyde phosphate to phosphoglyceric acid, is the limiting factor of the over-all reaction.

The above mechanism can be shown in simplified form as follows

- (a)  $\text{Glucose} + \text{ATP} \rightarrow \text{fructose-1,6-diphosphate}$   
 (b)  $\text{Glyceraldehyde phosphate} \rightleftharpoons \text{phosphoglyceric acid} + \text{ATP}$   
 (c)  $\text{Phosphoglyceric acid} \rightleftharpoons \text{lactic acid}$

It is clear that, if reaction (c) is limiting, a partial inhibition of reaction (b) will not be observed if reaction (b) plus (c) is measured, if reaction (a) plus (b) plus (c) is measured and reaction (a) becomes limiting because of its dependence on reaction (b), a pronounced inhibition may result

It is felt that such considerations have a bearing on a number of observations on the effect of inhibitors reported in the literature. For instance, it has been stressed by Shon (11) that concentrations of iodoacetic acid which suffice to inhibit anaerobic glycolysis are ineffective aerobically. Since glyceraldehyde phosphate dehydrogenase among the glycolytic enzymes is most highly susceptible to this inhibitor, it might be assumed that its partial inhibition may be observed only under anaerobic conditions, while aerobically ATP can be produced from oxidation of pyruvic acid, masking the inhibition. This explanation would eliminate the necessity to postulate other pathways of glucose breakdown.

The possible relation of the inactivating factor to a proteolytic enzyme has been considered previously (1). A serious objection to this assumption lay in the apparent specificity of the reaction for one of the glycolytic enzymes only. It has now been demonstrated that the inactivating factor of brain homogenate affects at least one additional glycolytic enzyme, although to a lesser extent. Since both trypsin and cathepsin inhibit glycolysis in the same manner as the inactivating factor, it must be assumed that the glycolytic enzymes vary considerably in their susceptibility to proteolytic enzymes. These observations with known proteolytic enzymes shift the problem of specificity from the inactivating factor to the substrate. The importance of substrate specificity has been stressed by Bergmann and Fruton (12) in their studies on hydrolysis of synthetic peptides. A better knowledge of the susceptibility of different glycolytic enzymes to the series of known proteolytic enzymes, as well as of their protection from proteolysis by substrates and coenzymes, may throw some light on the construction of the enzyme protein molecule and its active centers.

#### SUMMARY

1. The inhibiting effect of ferrous sulfate on brain glycolysis has been localized to the coupled phosphorylation catalyzed by the phosphoglyceraldehyde-oxidizing enzyme. Partial inhibition of this enzyme leads to in-

hibition of the synthesis of ATP which is necessary for the formation of fructose-1,6-diphosphate from glucose

2 The phosphoglyceraldehyde-oxidizing enzyme prepared from yeast or muscle restores the glycolytic activity to a brain homogenate which has been inactivated by ferrous sulfate or by the addition of proteolytic enzymes

3 The inactivating factor loses its activity after dialysis against 0.1 M KCN for 5 days. Full reactivation can be accomplished by the addition of ferrous sulfate and cysteine

4 The interpretation of the effect of inhibitors on complex reactions involving several enzymes is discussed

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# THREONINE-SERINE ANTAGONISM IN SOME LACTIC ACID BACTERIA

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WITH THE TECHNICAL ASSISTANCE OF MELBA BREEDLOVE SAMPLE

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Various investigators have reported antagonism between amino acids in the nutritional requirements of microorganisms. Snell and Gurard (1) have reported the inhibitory effect of glycine, serine,  $\beta$ -alanine, and threonine on the utilization of alanine. Gladstone (2), using *Bacillus anthracis*, found that certain amino acids prevented growth when added singly to a medium capable of supporting growth without them. He found valine to be inhibitory to leucine utilization and vice versa. Similar interrelationships were found for valine and threonine, and threonine and serine.

In preliminary investigations by the authors it was noted that serine assays with *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Streptococcus faecalis* R, and *Leuconostoc mesenteroides* P-60 varied greatly. Standard serine curves by these four organisms were characterized by having a lag section in the curve at low concentrations of serine, and variation in results seemed to follow the extent of the initial lag section of the curve, i.e., the assay values obtained with the organism having the greatest lag in the standard serine curve varied over a wider range than assay values obtained with those organisms exhibiting the smallest lag in the standard curve.

From the work of Gladstone (2) with *Bacillus anthracis* and from the sigmoidal shape of the standard serine curves, it was thought possible that the lag in the standard curve was due to the inhibition of serine utilization by threonine. This paper presents the results of experiments in a study of the inhibitory effect of threonine on serine utilization and also the inhibitory effect of serine on threonine utilization by some lactic acid bacteria.

## Procedure

Stock cultures of *Lactobacillus delbrueckii* 9595, *L. casei*, *Streptococcus faecalis* R (formerly known as *Streptococcus lactis* R), and *Leuconostoc mesenteroides* P-60 were carried on a solid medium containing 1.5 per cent agar, 1 per cent glucose, and 1 per cent yeast extract. *L. arabinosus* 17-5 was carried on a solid medium containing 0.8 per cent agar, 1 per cent peptonized milk, 1 per cent tryptone, and 200 ml of filtered tomato juice per liter of medium (3). The inoculum medium of Stokes and Gunness (4) was used



for all the organisms except *L. arabinosus*. The tomato juice medium given above with the agar omitted was used for inoculum media for this organism.

The metabolites to be studied (adjusted to pH of the medium) were added to tubes and the volume was adjusted to 5 ml with distilled water. 5 ml of appropriate double strength media were then added. The tubes were

TABLE I

*Composition of Leuconostoc mesenteroides, Media A\* and B*

Constituent	Final concentration per 10 ml tube		Constituent	Final concentration per 10 ml tube	
	Medium A	Medium B		Medium A	Medium B
	mg	mg		mg	mg
L-Arginine monohydrochloride	2	1	Adenine	0.12	0.10
DL-Alanine	10	2	Guanine	0.12	0.10
DL-Aspartic acid	20	4	Uracil	0.12	0.10
L-Cystine	2	1	KH <sub>2</sub> PO <sub>4</sub>	10	10
Glycine	1	1	K <sub>2</sub> HPO <sub>4</sub>	10	10
DL-Glutamic acid	8	4	MgSO <sub>4</sub> 7H <sub>2</sub> O	2	2
L-Histidine monohydrochloride	1	1	NaCl	0.10	0.10
DL-Isoleucine	2	2	MnSO <sub>4</sub> 4H <sub>2</sub> O	0.10	0.10
DL-Leucine	2	2	FeSO <sub>4</sub> 7H <sub>2</sub> O	0.10	0.10
L-Lysine monohydrochloride	2	1		γ	γ
DL-Methionine	1	2	Biotin	0.05	0.01
DL-Norleucine		2	Calcium pantothenate	20	2
L-Proline	1	1	Choline chloride		25
DL-Phenylalanine	1	2	Folvite†	0.04	0.04
L-Tryptophan	0.5	1	Inositol		25
L-Tyrosine	1	1	Nicotinic acid	20	2
DL-Valine	2	2	p-Aminobenzoic acid	0.01	0.01
Glucose	200	200	Pyridoxine hydrochloride	16	16
Sodium acetate (anhydrous)	120	60	Riboflavin	20	2
NH <sub>4</sub> Cl	60	30	Thiamine hydrochloride	10	5

\* Lyman and Kuiken, unpublished.

† Lederle Laboratories Division, American Cyanamid Company.

plugged with non-absorbent cotton and autoclaved at 15 pounds pressure for 10 minutes. After being cooled, each tube was inoculated with 1 drop of suspension of organisms which was prepared by washing a 10 ml. 18 to 24 hour culture twice with physiological saline, and then diluted to a final volume of 100 ml. The tubes were placed in a water bath at 35° and incubated for 96 hours for *Leuconostoc mesenteroides* and 72 hours for the other organisms. At the end of the incubation period, the lactic acid produced

was titrated with 0.1 N NaOH to pH 7.0, as determined by an indicating pH meter. The DL form of both serine and threonine was used in all the work presented. All concentrations of serine and threonine given in this paper refer to the DL form unless otherwise designated.

The medium of Stokes, Gunness, Dwyer, and Caswell (5) was used for *Lactobacillus delbrueckii* with the exception that pyridoxine hydrochloride (20  $\gamma$  per 10 ml tube) was used instead of pyridoxamine. For *Streptococcus*

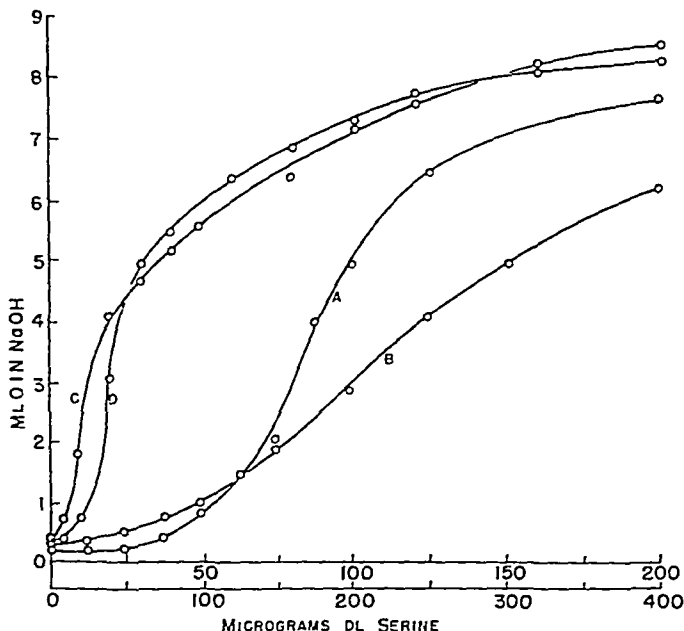


FIG 1 Standard serine curves. Curve A, *Lactobacillus delbrueckii*, 0 to 400  $\gamma$  of serine, Curve B, *Lactobacillus casei*, 0 to 400  $\gamma$  of serine, Curve C, *Streptococcus faecalis*, 0 to 200  $\gamma$  of serine, and Curve D, *Leuconostoc mesenteroides*, 0 to 200  $\gamma$  of serine.

*faecalis*, Medium II of Baumgarten, Mather, and Stone (6) was employed with the xanthine omitted. The *L. casei* medium as described by McMahon and Snell (7) with xanthine omitted was used for this organism. The media for *Leuconostoc mesenteroides* are given in Table I. Medium A of Table I is from unpublished work of Kuken and Lyman and was used in obtaining data for *Leuconostoc mesenteroides* in Figs 1 and 5. Medium B was employed for the other experiments with this organism. The medium of Lyman *et al* (3) was used for *L. arabinosus* with the omission of the tomato eluate and the addition of folic acid.

### Results

Standard serine curves with their characteristic lag at low levels of serine are shown in Fig 1 for four serine-requiring lactic acid bacteria. Such

curves are obtained in the presence of 2 mg of threonine per 10 ml tube, which is the amount of this metabolite present in basal media used for serine assays (4, 6) The lag section of Curves A and B, extending to 100  $\gamma$ , is approximately ten times greater than the lag section of Curves C and D

The inhibitory effect of threonine on the utilization of serine and the resulting lag in the standard serine curves are clearly demonstrated in Figs 2 to 5 *Lactobacillus delbrueckii* (Fig 2) and *L casei* (Fig 3) do not require threonine for growth and the omission of this metabolite from the media for these two organisms completely removes the lag in the growth curves

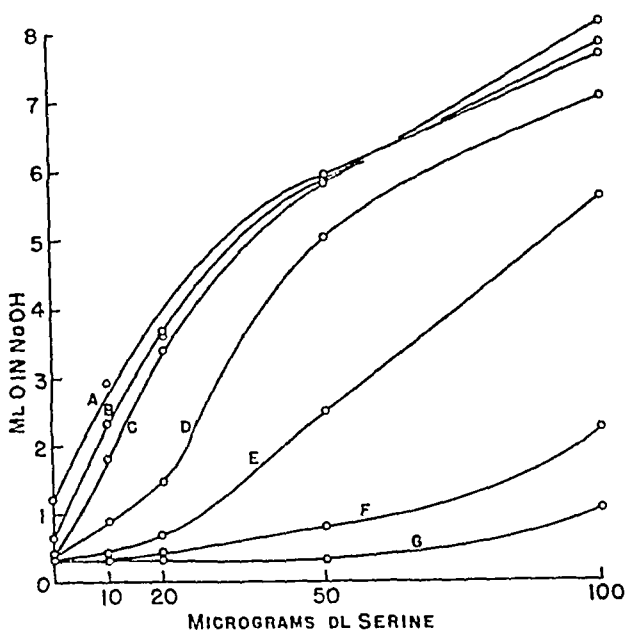


FIG 2 Effect of threonine on serine utilization by *Lactobacillus delbrueckii* Curve A, no threonine, Curve B, 100  $\gamma$  of DL-threonine per tube, Curve C, 200  $\gamma$  of DL threonine per tube, Curve D, 500  $\gamma$  of DL-threonine per tube, Curve E, 1000  $\gamma$  of DL threonine per tube, Curve F, 2000  $\gamma$  of DL-threonine per tube, Curve G, 4000  $\gamma$  of DL-threonine per tube

With standard serine Curves A, B, and C of Fig 2, obtained in the presence of 0, 100, and 200  $\gamma$  of threonine, it is to be noted that Curve A falls below Curves B and C for higher growth. This would suggest that threonine is stimulatory for *L delbrueckii* at higher growth levels. However, in other experiments this was not apparent and was not noticed with *L casei* (Fig 3)

*Streptococcus faecalis* and *Leuconostoc mesenteroides* both require threonine and it is therefore impossible to remove the lag sections completely by the omission of threonine. From Figs 4 and 5 for these two organisms, it is apparent that the lag is reduced by diminishing the threonine, but it seems that for *L mesenteroides* and possibly *Streptococcus faecalis* some other fac-

tors are responsible for some of the lag. These results with *L. mesenteroides* are similar to those of Horn, Jones, and Blum (8) in which they found it impossible to remove the lag completely from the standard threonine curves by lowering the serine content of the basal medium.

**Inhibition Ratios of Threonine to Serine**—A quantitative study of the inhibition of serine utilization is given in Table II. For this study the "antibacterial index" method of McIlwain (9) was used. This particular approach has been extensively used by Shive and coworkers (10-12) in studying competitive analogue-metabolite growth inhibitions. For data in Table II a wide range in concentration of threonine was used and serine was added to give the ratios as indicated. The ratio of threonine to serine at which the

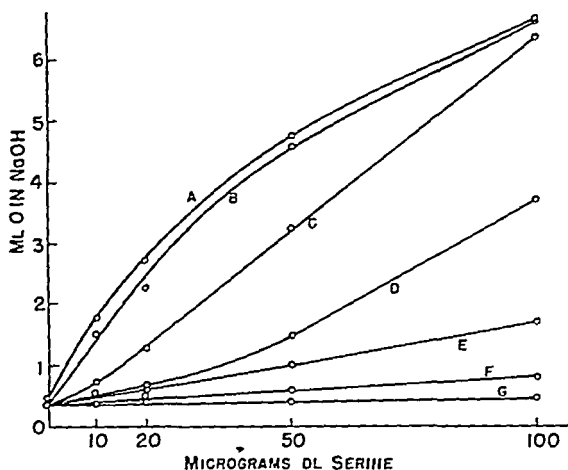


FIG 3 Effect of threonine on serine utilization by *Lactobacillus casei*. Curve A, no threonine, Curve B, 100  $\gamma$  of DL-threonine per tube, Curve C, 200  $\gamma$  of DL-threonine per tube, Curve D, 500  $\gamma$  of DL-threonine per tube, Curve E, 1000  $\gamma$  of DL-threonine per tube, Curve F, 2000  $\gamma$  of DL-threonine per tube, Curve G, 4000  $\gamma$  of DL-threonine per tube.

titrations are equal to the blank titrations (no serine added) is taken as the "antibacterial index" or inhibition ratio. For *Lactobacillus delbrueckii* the inhibition ratio is between 100 and 200. At the ratio of 100 there was growth at higher levels of threonine and serine. This indicated that at this ratio there was still serine present for growth above the ratio of inhibition. At low levels this amount of serine was so small that no response could be detected. However, at a ratio of 200 the inhibition was complete for the concentrations of threonine and serine used. An experiment repeated with the 100 and 200  $\gamma$  serine levels, with varying amounts of threonine, gave in each case a ratio of 150. *Lactobacillus casei* was completely inhibited at a ratio of between 50 and 100, as shown by Table II. A repetition of the experiment with 100 and 200  $\gamma$  of serine and varying concentrations of

threonine gave an inhibition ratio of 75. Inhibition ratios greater than 1000 for *Leuconostoc mesenteroides* and 2000 for *Streptococcus faecalis* are in-

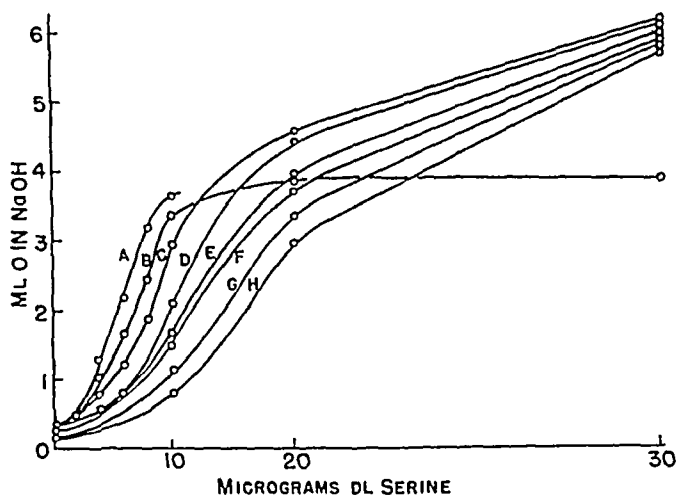


FIG 4 Effect of threonine on serine utilization by *Streptococcus faecalis*. Curve A, 25  $\gamma$  of DL-threonine per tube, Curve B, 50  $\gamma$  of DL-threonine per tube, Curve C, 100  $\gamma$  of DL-threonine per tube, Curve D, 200  $\gamma$  of DL-threonine per tube, Curve E, 500  $\gamma$  of DL-threonine per tube, Curve F, 1000  $\gamma$  of DL-threonine per tube, Curve G, 2000  $\gamma$  of DL-threonine per tube, Curve H, 4000  $\gamma$  of DL-threonine per tube.

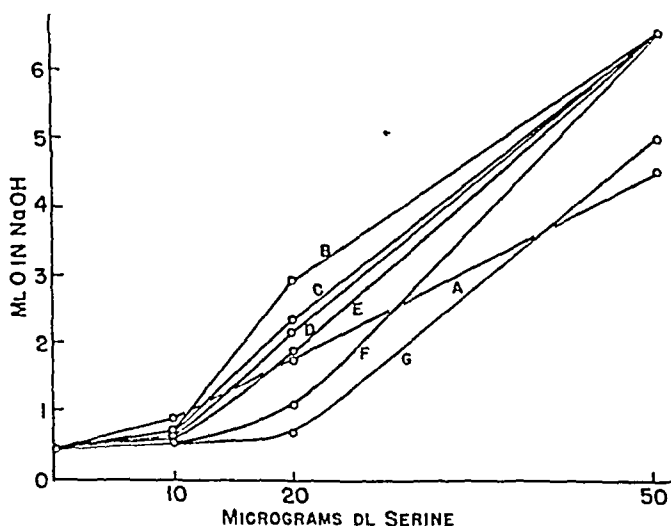


FIG 5 Effect of threonine on serine utilization by *Leuconostoc mesenteroides*. Curve A, 50  $\gamma$  of DL-threonine per tube, Curve B, 100  $\gamma$  of DL-threonine per tube, Curve C, 200  $\gamma$  of DL-threonine per tube, Curve D, 500  $\gamma$  of DL-threonine per tube, Curve E, 1000  $\gamma$  of DL-threonine per tube, Curve F, 2000  $\gamma$  of DL-threonine per tube, Curve G, 4000  $\gamma$  of DL-threonine per tube.

dicted by Table II. By extrapolation of this data, ratios of 1100 to 1400 and 2600 to 2800 are obtained. Analogous experiments with 10 and 20  $\gamma$

TABLE II  
Inhibition of Serine Utilization by Threonine

Organism	Threonine	Titration, ml of 0.05 N NaOH				
		Ratio of threonine to serine				
		No serine	200	100	50	25
<i>Lactobacillus delbrueckii</i>	mg					
	0.20	0.70	0.60	0.70	1.05	1.00
	0.50	0.60	0.60	0.60	0.80	0.80
	1.00	0.50	0.55	0.50	0.80	1.36
	2.00	0.52	0.52	0.50	0.74	0.92
	4.00	0.50	0.54	0.55	0.84	1.05
	10.00	0.55	0.60	0.58	1.05	1.50
	20.00	0.56	0.60	0.65	1.20	2.55
	40.00	0.70	0.70	1.20	2.28	13.75
<i>Lactobacillus casei</i>		Ratio of threonine to serine				
		No serine	100	50	25	10
	0.20	0.85	0.71	0.83	0.91	1.00
	0.50	0.70	0.70	0.82	0.85	1.45
	1.00	0.70	0.70	0.80	0.81	2.30
	2.00	0.70	0.70	0.61	0.80	1.68
	4.00	0.68	0.66	0.71	0.67	2.37
	10.00	0.68	0.68	0.80	0.84	3.60
	20.00	0.70	0.72	0.90	1.13	9.08
<i>Leuconostoc mesenteroides</i> P-60		Ratio of threonine to serine				
		No serine	1000	400	200	100
	0.50	0.14	0.80	0.94	0.90	0.94
	1.00	0.72	1.00	1.00	0.97	3.78
	2.00	0.80	0.89	0.95	1.33	4.17
	4.00	0.78	0.81	1.15	2.00	8.72
	10.00	0.79	0.90	2.00	7.80	10.80
	20.00	0.85	1.10	3.96	13.10	14.80
<i>Streptococcus faecalis</i> R		Ratio of threonine to serine				
		No serine	2000	1500	1000	800
	2.00	0.04	0.14	0.34	0.48	0.59
	4.00	0.04	0.34	0.54	0.34	0.62
	10.00	0.00	0.56	0.84	1.32	2.09
	20.00	0.00	0.84	1.51	3.49	4.14
	40.00	0.00	1.84	3.34	7.38	8.84

of serine gave inhibition ratios of 1500 for *Leuconostoc mesenteroides* and 2000 to 4000 for *Streptococcus faecalis*

*Inhibition Ratios of Serine to Threonine*—If threonine, because of its similarity in structure to serine, can block the utilization of serine, it should also be feasible that serine could block the utilization of threonine. Data in Table III clearly demonstrate that serine can effectively inhibit the utilization of threonine by some threonine-requiring lactic acid bacteria that have been used for the assay of threonine. An inhibition ratio of serine to threonine of approximately 600 is obtained for *Streptococcus faecalis* by interpolation and extrapolation of data given in Table III. By the same method, the best values indicate an inhibition ratio of 200 for *Leuconostoc mesenteroides* and between 400 and 600 for *Lactobacillus arabinosus*.

TABLE III  
*Inhibition of Threonine Utilization by Serine*

Organism	Threo- nine per tube	Serine per tube, mg										
		0	0.4	1.0	2.0	4.0	10.0	20.0	40.0	60.0	80.0	100.0
		Titration, ml of 0.1 N NaOH										
	$\gamma$											
<i>Streptococcus faecalis</i>	20	0.95	2.86	2.85	2.82	2.74	1.34	0.0				
	100	0.62	7.60	7.42	7.30	7.30	6.30	5.50	4.12	0.78		
	200	0.56	8.62	8.70	8.50	8.52	8.30	8.25	7.70	6.45	4.20	2.00
	20	0.30	0.91	0.30	0.30	0.26	0.32					
<i>Leuconostoc mesenteroides</i> P 60	100	0.32	4.18	4.02		3.85	0.84	0.33	0.32			
	200	0.68	5.80	5.20	4.80	3.68	3.30	3.18	0.30	0.30	0.35	
<i>Lactobacillus arabinosus</i>	100	10.10			6.35	4.82	2.81	2.04	1.81	1.70	1.72	1.72

DISCUSSION

The antagonistic effect of threonine on the utilization of serine has some implications as to the reliability of the microbiological determination of serine. The technique of heavily dosing the medium with all constituents, other than the one to be assayed, is usually sufficient to eliminate the effect of metabolites added as samples. However, with *Lactobacillus delbrueckii* and *Lactobacillus casei*, the addition of excess threonine (2 mg per 10 ml tube) is not desirable. The large lag section in the standard serine curve by these two organisms obtained in the presence of high concentration of threonine is objectionable because in the lag section the growth response to added serine is relatively small. This decreases the sensitivity and limits the accuracy of assay values taken in the lag section.

Also in serine assays with *Lactobacillus delbrueckii* and *Lactobacillus casei*, the effect of threonine added as a sample is important even in the presence of 2 mg of threonine per tube. Curve F (2 mg of threonine per tube) and Curve G (4 mg of threonine per tube) of Fig 2 demonstrate adequately that the addition of threonine, in excess of the 2 mg usually used in assay procedure, causes a decrease in titration at a given level of serine. For example, with Curves F and G there is a difference in titration of 0.5 ml at 50  $\gamma$  of serine and 1.2 ml at 100  $\gamma$  of serine. Experimental data not shown, but which represent an extension of these same two curves, Curves F and G, gave differences in titration of 4.25 ml at 200  $\gamma$  of serine and 3.75 ml at 300  $\gamma$  of serine. It is true that these differences were obtained by adding 2 mg of threonine in excess of the amount used in Curve F, but Table IV

TABLE IV

Comparison of Amount of Threonine Added by Several Samples in Assay for Serine by *Lactobacillus delbrueckii*

Sample	L-Serine	L-Threonine	Assay range DL-serine	Sample for assay range	L-Threonine added as sample for assay range
	per cent	per cent	$\gamma$	mg	$\gamma$
Dried cheese whey	0.04 (6)	1.9 (6)	0-400	0-50	0-950
Casein	6.8 (6)	4.5 (6)	0-400	0-3	0-135
Silk fibroin	11.4 (13)	1.3 (13)	0-400	0-2	0-26
	14.5 (4)				

The figures in parentheses refer to the bibliography

shows that such conditions are possible in the serine assay by *Lactobacillus delbrueckii*.

Table IV gives three samples that vary in the ratio of the threonine to the serine content. It can be noted from Table IV that with such a sample as dried cheese whey, in which the threonine content is approximately five times greater than the serine content, a maximum of 950  $\gamma$  of L-threonine would be added in an assay range of 0 to 400  $\gamma$  of serine (Curve A, Fig 1). This quantity of threonine added as a sample approaches very closely the difference in L-threonine content of the media used in obtaining Curves F and G, Fig 2. Such additions of threonine would have a pronounced effect on the response of the organism to serine and as a result would give low assay values which would become increasingly lower as the amount of the sample was increased. Silk fibroin on the other hand has a much higher serine content than threonine and therefore the amount of threonine added would be very small, as is shown by Table IV. Thus with this sample the effect of added threonine as a sample would be at a minimum.



Casein is more representative of most of the samples on which serine and threonine values have been reported (6, 13). Although most of these samples contain a little more serine than threonine, it is evident from Table IV and Fig. 2 that the additions of threonine as a sample would have the same effect on the assay as with dried cheese whey, only to a lesser extent.

The data presented indicate that a good assay procedure for serine, in samples containing no threonine, would be the use of *Lactobacillus delbrueckii* with a basal medium free of threonine.

The use of *Streptococcus faecalis* or possibly *Leuconostoc mesenteroides* shows more promise of being adapted to a serine assay than any of the other organisms mentioned in this paper, when the effect of factors other than threonine is more clearly understood. With *Streptococcus faecalis* the lag section is relatively short and the upper limit of the assay range is low. This allows the use of small quantities of sample which minimizes the effect of threonine added as a sample. These possibilities are being investigated in this laboratory and will be reported later.

#### SUMMARY

Data have been presented to show the antagonistic effect of threonine on the utilization of serine. Increasing amounts of threonine added to basal media have been shown to cause a decrease in growth at a given level of serine. Ratios of threonine to serine of approximately 150, 75, 1100 to 1400, and 2000 to 4000 completely prevented growth of *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Leuconostoc mesenteroides*, and *Streptococcus faecalis* under the conditions presented. Implications of the antagonistic effect of threonine on serine utilization as related to the microbiological assay methods for serine have been discussed.

A few data have also been presented concerning the antagonistic effect of serine on the utilization of threonine. Ratios of serine to threonine of 600, 200, and 400 to 600 have been shown to inhibit effectively the growth of *Streptococcus faecalis*, *Leuconostoc mesenteroides*, and *Lactobacillus arabinosus*.

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# THE EFFECT OF SURFACE-ACTIVE SUBSTANCES ON THE FUCHSIN REACTION OF HIGHER FATTY ALDEHYDES\*

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Higher fatty aldehydes are present in considerable quantity in the lipid fraction of muscle and brain and may be intermediates in lipid metabolism (1, 2). The meager information concerning the quantitative distribution of the higher fatty aldehydes in tissues has been acquired exclusively by quantitative evaluation of the Schiff reaction, as in the procedure of Feulgen and Grünberg (3) or modifications of it (4). Serious doubt as to the reliability of the method arose when it was found that added palmitaldehyde or stearaldehyde or their acetals could not be estimated quantitatively in tissue extracts (4).

The experiments reported in this paper show that the result of the quantitative fuchsin reaction for the determination of higher fatty aldehydes depends to a large degree on the presence of surface-active lipides in the tissue extract. Naturally occurring lipides or synthetic surface-active agents inhibit the color development if added at the beginning of the reaction, and destroy the color already formed if added later. This effect of surface-active agents can be suppressed to a large degree by reducing the water content of the medium through the use of a high concentration of acetic acid.

## EXPERIMENTAL

The Schiff reaction was employed in three forms, two at low and one at high concentrations of acetic acid. In all experiments palmitaldehyde glyceryl acetal was used as reference substance (4).

*Reactions at Low Acetic Acid Concentrations (Reactions S<sub>F</sub> and S<sub>F</sub> HCl)*—The Schiff reaction for the determination of higher fatty aldehydes as carried out previously in our laboratory (4) differed in three respects from the procedure used by Feulgen and Grünberg (3). (a) 1 ml. of 1 N HCl was added to the reaction mixture consisting of 10 ml. of fuchsin reagent, mercuric chloride solution, and 1 ml. of glacial acetic acid containing the compounds or tissue components to be tested. It was found that increased

\* The higher fatty aldehydes, IV. This work was supported by grants from the Josiah Macy, Jr., Foundation and the United States Public Health Service.

acidity resulted in a greater precision (b) The color was developed at 37° for 18 to 24 hours (c) The color complex was extracted with capryl (4, 5) instead of amyl alcohol (3) In the present experiments this procedure (Reaction S F HCl) was compared with a procedure (Reaction S F) in which no HCl was added The reaction mixtures contained 8.9 per cent (Reaction S F) and 8.1 per cent (Reaction S F HCl) acetic acid respectively

*Reaction at High Acetic Acid Concentration (Reaction S A A)*—2 gm of basic fuchsin (National Aniline Division) were dissolved in 50 ml of glacial acetic acid 10 gm of sodium bisulfite, 100 ml of 0.1 N HCl, and 50 ml of water were added in succession The reagent was used after it had stood for several hours The bisulfite did not decolorize the solution appreciably, the final reagent retained a reddish brown color To 1 ml of glacial acetic acid containing the compounds or tissue components to be tested 2 ml of glacial acetic acid and 1 ml of fuchsin reagent were added The color was developed in sealed glass tubes (9 mm inside diameter, 10 ml capacity) at 50° for 18 to 20 hours When cool, the sealed tubes were opened, 2 ml were transferred to a 25 ml graduated cylinder (glass-stoppered), and 10 ml of an aqueous solution were added, containing 5 gm of sodium bisulfite and 5 ml of concentrated HCl in 100 ml In the blank samples the color faded within 10 minutes to a light yellow The solutions were extracted with 10 ml of capryl alcohol exactly 10 minutes after the addition of the sulfite solution, and the alcoholic solution was cleared by centrifuging as described previously (4)

*Substrates*—The preparation of palmitaldehyde and stearylaldehyde and then acetals was described previously (4) As synthetic surface-active substances the non-ionic detergents, Tweens and Spans, of the Atlas Powder Company (mono- and polyesters of sorbitan with long chain fatty acids and then polyalkylene derivatives) were used These substances produced small and consistent color values The crude egg yolk phosphatides were prepared according to the method of Feulgen and Grunberg (3) and dried to constant weight The samples gave fuchsin color values (Reaction S F HCl) corresponding to as much as 720 mg of palmitaldehyde per 100 gm of lipid In the experiments with brain extract the residue of an alcohol-ether extract of finely minced brain was dissolved in the appropriate amount of glacial acetic acid

In all experiments reported in this paper the color density was determined with a Coleman junior spectrophotometer, model 6, in cuvettes No 6-302 at 515 mμ The values obtained in Reaction S A A were doubled, since only half of the reaction mixture was extracted with capryl alcohol

#### RESULTS AND DISCUSSION

The addition of Span 20, egg yolk phosphatides, or brain lipides to palmitaldehyde or its glyceryl acetal resulted in an inhibition of the color de-

velopment if the Schiff reaction was carried out in a medium of approximately 90 per cent water (Reactions S F and S F HCl) (Table I) By carrying out the Schiff reaction in a medium containing 80 per cent acetic

TABLE I

*Schiff Reaction of Palmitaldehyde and Its Glyceryl Acetal in Presence of Lipides and Synthetic Surface-Active Agents*

Source of aldehyde	Amount	Addition	Amount	Schiff reaction* optical density			Analytical recovery		
				Reaction S F	Reaction S F HCl	Reaction S A A †	Reaction S F	Reaction S F HCl	Reaction S A A
	$\gamma$		mg				per cent	per cent	per cent
Palmitaldehyde	30				0 11	0 24			
	30	Span 20	4		0 02	0 23		18	96
	30	" 20	8		0	0 22		0	93
	40			0 16	0 16	0 24			
	40	Span 20 ‡	20	0 09	0 03	0 25	56	20	105
	35					0 26			
	35	Phosphatides	16			0 20			77
	20			0 12	0 09				
	20	Phosphatides	20	0 05	0 02		40	22	
	20				0 07	0 12			
Palmitaldehyde glyceryl acetal		Brain lipides	14§		0 07	0 24			
	20	" "	14		0 10	0 32		44	66
	82			0 31	0 28	0 68			
	82	Span 20	20	0 02	0 05	0 64	6	18	94
	30					0 26			
	30	Phosphatides	15			0 26			100
	40				0 16				
	40	Phosphatides	1		0			0	
	61					0 54			
	61	Brain lipides	11			0 32			
	61	" "	11			0 78			85

\* In all experiments in which Span 20 or egg phosphatides were added the values are corrected for the densities given by these substances alone

† Optical density for palmitaldehyde glyceryl acetal 20 4  $\gamma$ , 0 17, 40 8  $\gamma$ , 0 35, 61 2  $\gamma$ , 0 52, 81 6  $\gamma$ , 0 66, 102  $\gamma$ , 0 82, for palmitaldehyde 20 2  $\gamma$ , 0 15, 40 5  $\gamma$ , 0 30, 81  $\gamma$ , 0 54, 101  $\gamma$ , 0 66

‡ Span added 5 hours after the start of the reaction

§ Weight of wet brain

acid (Reaction S A A) the effect of the surface-active agents was minimized and the recoveries of added aldehyde or acetal amounted to 66 to 100 per cent. The recovery of total color resulting from the aldehyde or acetal plus that from various compounds which were added amounted in Reactions S F and S F HCl to 0 to 74 per cent and in Reaction S A A to 88 to 100 per cent.

The color reaction at high acetic acid concentration is approximately twice as sensitive as that carried out at low acetic acid concentrations. Of the latter reactions the one with the higher acidity (Reaction S F HCl) is slightly less sensitive and more susceptible to the action of surface-active agents than the one with lower acidity (Reaction S F).

In agreement with previous observations, equivalent quantities of different aldehydes or of the same aldehyde on different days did not yield the same color density. The same aldehyde sample tested in different concentrations does not follow Beer's law (*cf* foot-note, Table I) probably because of the difficulty in obtaining monomeric aldehydes, and therefore the results of experiments carried out on these substrates are variable. With acetal a better linear relation is obtained (*cf* foot-note, Table I).

In an experiment reported in Table I, 1 mg of egg yolk phosphatide inhibited completely the color developed by 40  $\gamma$  of acetal (Reaction S F HCl). However, when 0.5 mg of phosphatide was added, definite inhibition of color development was found in some experiments, but in others the color values coincided (within the error of the method) with the control value of acetal alone. This finding may explain the result reported previously (4) that addition of 1 mg of egg yolk phosphatides, prepared according to Feulgen and Grunberg, does not affect the 18 hour color value of acetal. In that experiment the phosphatide preparation used was not dried to constant weight and the actual amount may have been considerably less than 1 mg.

The effect of surface-active agents can also be demonstrated on the aldehydes present in tissue extracts. 20 mg of Tween 85 were added to a solution of brain lipides in glacial acetic acid. Measurements at different time intervals from 30 minutes up to 18 hours after mixing the reagents gave color values corresponding to 10 to 16 per cent of the simultaneously determined control values. Similar results were obtained in experiments in which Span 20 was used as the surface-active agent.

The addition of detergent after full color had developed with palmitaldehyde (Reaction S F HCl) caused fading. On the other hand if Reaction S A A was used, full color was obtained when the detergent was added 5 hours after mixing of the reagents (Table I).

The effect of surface-active agents on the Schiff reaction of the higher fatty aldehydes in aqueous medium supports the point of view expressed previously (4), that analytical results obtained with the fuchsin method probably do not represent the true concentrations of higher fatty aldehydes in tissue extracts. The fact that consistent values have been obtained may be a reflection of relatively constant ratios between higher fatty aldehydes and surface-active lipides. An apparent variation, found by the fuchsin method in aqueous media, in the aldehyde concentrations in a tissue under

physiological or pathological conditions may be the result of a change in concentration of aldehydes, of surface-active lipides, or of both (5, 6). The form in which the aldehydes are present in the extracts is not known with certainty, and the lipide composition occurring in an extract obtained from tissues with an organic solvent cannot be duplicated experimentally. It is, therefore, difficult to decide how well model experiments with detergents, free aldehydes, and acetals approximate conditions in tissue extracts. But it appears that the new procedure elaborated for the use of the Schiff reaction as presented in this paper may eliminate one of the potential errors in the determination of the higher fatty aldehydes in tissue extracts.

#### SUMMARY

Naturally occurring lipides and synthetic surface-active substances inhibit the color development of the higher fatty aldehydes and their acetals in the fuchsin test as proposed by Feulgen and used in the original or modified form by others. If the surface-active substances are added after color has developed, rapid fading occurs. Addition of synthetic detergents in the determination of aldehydes present in tissue lipides also suppresses the development of color to a marked degree. These findings cast serious doubt on the usefulness of the fuchsin method for the quantitative determination of the higher fatty aldehydes as carried out with the Feulgen method or its modifications.

The effect of surface-active, naturally occurring or synthetic agents is suppressed to a large degree if the Schiff reaction is carried out in a medium containing a high concentration of acetic acid.

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# THE PEPTIDASES OF SKELETAL, HEART, AND UTERINE MUSCLE\*

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Early investigators of peptidases ascribed the hydrolysis of simple peptides to a relatively small number of enzymes. In recent years, it has become evident that there must be many different peptidases whose specificity is dependent on the nature of the constituent amino acids as well as on the presence of free amino or carboxyl groups. Most of the early studies were confined to a few animal tissues with intestinal mucosa as the main source of these enzymes. It is obviously desirable to examine other tissues for the presence of proteolytic enzymes.

In this study, it will be shown that peptidases of high activity are present in skeletal, heart, and uterine muscle. In general, these enzymes closely resemble those previously studied from intestinal mucosa (Smith and Bergmann (1)) and from skin, lung, and serum (Fruton (2)). All of these tissues appear to contain mainly enzymes of the type which have been designated exopeptidases, *z. e.*, enzymes which are capable of hydrolyzing peptide bonds adjacent to free terminal amino and carboxyl groups. Endopeptidases which are capable of hydrolyzing peptide linkages which are not adjacent to free terminal amino and carboxyl groups have not been found in significant amounts in these tissues.

## *Peptidases of Rabbit and Rat Skeletal Muscle*

Tables I and II present some of the data obtained with aqueous extracts of rabbit and rat muscle. The hydrolysis of L-leucylglycine (LG) is strongly activated by the presence of 0.001 M  $\text{MnSO}_4$  and is probably due to the presence of a leucine aminopeptidase similar to those already described in other tissues (1-3). The hydrolysis of the tripeptide L-leucylglycylglycine (LGG) is greater than that of the dipeptide and is also activated by  $\text{Mn}^{++}$ . The hydrolysis of the tripeptide appears to be caused by the concurrent action of the leucine aminopeptidase, and by a distinct enzyme which is not activated by a metal but which also hydrolyzes diglycylglycine (GGG) and other tripeptides (Smith and Bergmann (1), Fruton (2)). Za-

\* This investigation was aided by a grant from the United States Public Health Service.

mecnik, Stephenson, and Cope (4) have found that extracts of dog muscle hydrolyze LGG

TABLE I  
*Proteolytic Enzymes of Rabbit Muscle*

The enzyme preparation was a crude aqueous extract. The tests were performed at 40° in a diethylbarbiturate (veronal) buffer at pH 7.8 to 8.0 or in acetate buffer at the acid pH values.

Substrate	Protein N per cc test solution	pH	Time	Hydrolysis		
				No acti- vator	0.001 M Mn <sup>++</sup>	0.003 M cysteine
	mg		hrs	per cent	per cent	per cent
L-Leucylglycine	0.226	8.0	2.5	8	73	
			3.5	12	95	
			6	29	100	
			24	101		
L-Leucylglycylglycine	0.226	7.8	0.5	9	11	
			2.5	33	83	
			3.5	54	98	
			6	92	109	
Diglycylglycine	0.251	8.0	2	20	36	
			24	81	145	
L-Prolylglycine	0.226	7.8	23	7	29	
			51	17	62	
Glycyl-L-proline	0.226	8.0	2		41	
			21	38	103	
Glycyl-L-leucine	0.340	7.8	45	65		
			7	28	26	
			24	51	65	
Glycylglycine	0.251	8.0	48	67	98	
Glycyl-L-phenylalanine	0.226	7.9	21	10	86	
			45	18		
			45	0		
Benzoyl-L-argininamide	0.226	4.0	45	0		
		5.0	26	0		2
		8.0	26	10		8
Carbobenzoylglycyl-L-phenylalanine	0.226	5.0	26	14		9
		7.9	45	5		
Carbobenzoyl-L-isoglutamine	0.226	5.2	26	9		5
		7.9	45	1		
Carbobenzoyl-L-glutamyl-L-tyrosine	0.226	6.2	45	2		
		5.4	26	4		3
Benzoylglycinamide	0.226	8.0	45	2		
		5.2	45	2		

Prolinase (hydrolysis of L-prolylglycine) and prolidase (hydrolysis of glycyl-L-proline) are also present in muscle extracts, and like the similar enzymes of intestinal mucosa (1) are markedly activated by Mn<sup>++</sup> ions.

Many other simple dipeptides are hydrolyzed by crude extracts of muscle. The hydrolysis of glycylglycine is strongly activated by  $\text{Co}^{++}$  ions as well as

TABLE II

*Proteolytic Enzymes of Rat Muscle*

The experiments were performed with two different crude aqueous extracts of leg muscle (0.186 mg of protein N per cc, \* and 0.163 mg of protein N per cc †) at 40° in diethyl barbiturate buffer at pH 7.8 to 8.0 unless otherwise specified

Substrate	Time	Hydrolysis		
		No activator	0.001 M $\text{Mn}^{++}$	0.001 M $\text{Co}^{++}$
	hrs	per cent	per cent	per cent
L-Leucylglycine*	2	3	34	
	4	6	63	
	24	63	101	
L-Leucylglycylglycine*	2	34	59	
	4	62	91	
	24	104	106	
Diglycylglycine*	2	29	25	
	4	49	44	
	5	60	54	
	24	96	99	
L-Prolylglycine*	24	4	32	
	48	10	50	
Glycyl-L-proline*	24	19	26	
	24	54	79	
	48	66	93	
Glycylglycine†	3	19	37	83
	5		53	97
	24	34	96	102
Glycinamide†	48	11	13	9
Benzoylglycine (pH 8.0)†	48	2	0	0
" (" 4.0)†	48	0		
Benzoylglycylglycine†	48	0	1	0
Benzoylglycinamide (pH 8.0)†	24	3		0
" (" 5.0)†	24	3		1
Carbobenzoyglycyl-L-phenylalanine (pH 8.0)†	48	4		
" (" 5.4)†	48	-1		0†
Carbobenzoy-L-isoglutamine (pH 8.0)†	48	3		
" (" 5.4)†	48	0		0†
Benzoyl-L-argininamide (pH 8.0)†	48	3		
" (" 5.4)†	48	2		3†

† These test solutions contained 0.003 M cysteine

by  $\text{Mn}^{++}$ , activation by cobalt appears to be extremely specific for this peptide (5) Berger and Johnson (6) and Maschmann (7) have previ-

ously found that the splitting of dipeptides may be specifically activated by different metal ions. The specificity and some other properties of the glycylglycine-splitting enzyme will be reported elsewhere (5).

Most of the enzymes designated as cathepsins which have been found in spleen, liver, and kidney (8) are activated by agents such as cysteine, ascorbic acid, etc. Hydrolysis by muscle extracts of known synthetic substrates for catheptic enzymes has not been found to any significant degree. These include such activities as a carboxypeptidase (substrate, carbobenzyloxylglycyl-L-phenylalanine), and various endopeptidases which hydrolyze carbobenzoxy-L-isoglutamine (substrate for papain), benzoyl-L-argininamide (substrate for trypsin and similar enzymes), and carbobenzoxy-L-glutamyl-L-tyrosine (substrate for pepsin and similar enzymes). Fruton (2) has recently found that benzoylglycinamide is hydrolyzed by an enzyme present in human and rabbit sera. This activity has not been detected in muscle extracts. Hippuricase (which hydrolyzes benzoylglycine) is not present in these extracts.

The absence of large amounts of true proteases from our extracts is also indicated by the fact that little or no autolysis could be detected when extracts of the different tissues were incubated at 40° for 48 hours in buffers at pH 4.0 to pH 8.0, and in the presence of cysteine, or various metal ions. Such experiments served as controls for the observation of the hydrolysis of synthetic substrates.

Kies and Schwimmer (9) have described the presence of a cathepsin in calf muscle which has an optimum action near pH 3.5 as judged by the liberation of soluble tyrosine from hemoglobin. Our failure to detect significant amounts of autolysis in our preparations of rat and rabbit muscle may be due to a species difference, or to a much lower concentration of such enzymes in our filtered extracts.

#### *Peptidases of Rabbit Heart and Uterus*

Aqueous extracts of rabbit heart muscle and rabbit uterus (Table III) show the same kinds of enzymatic activities as do skeletal muscle extracts. In general, these extracts contain higher peptidase activities than those obtained from the skeletal muscle of this species. Carboxypeptidase and endopeptidase activities have not been detected in the extracts of heart or uterus. This was tested at pH 5.0 and pH 8.0 in the presence or absence of cysteine with carbobenzyloxylglycyl-L-phenylalanine, benzoyl-L-argininamide, carbobenzoxy-L-isoglutamine, and carbobenzoxy-L-glutamyl-L-tyrosine.

#### *Peptidases of Human Uterus*

Few investigations have been made on the peptidases of human tissues. Beiger and Johnson (10) prepared an extract of human duodenum and stud-

TABLE III

*Peptidases of Rabbit Heart and Rabbit Uterus*

The uterus (fresh weight, 28 gm ) was obtained from a rabbit 2 weeks post partum  
 The enzyme experiments were performed at 40° in veronal buffer at pH 7.8 to 8.0

Tissue	Substrate	Protein N per cc test solution	Time	Hydrolysis		
				No activator	0.001 M Mn <sup>++</sup>	0.001 M Co <sup>++</sup>
		mg	hrs	per cent	per cent	per cent
Heart	L-Leucylglycine	0.0552	2		39	
			5.5	13	84	
			24	87		
	L-Leucylglycylglycine	0.0552	1.5		30	
			2.5	13	51	
			5.5	40	99	
			24	104		
	Diglycylglycine	0.138	1.5	20		
			2	30		
			3	40		
			6.5	74		
	L-Prolylglycine	0.138	24	96		
			3		14	
			6.5		26	
	Glycyl-L-proline	0.138	24		64	
			3		9	
			6.5		30	
Uterus	L-Leucylglycine	0.0448	24		80	
			24	9		77
			3		13	
	Glycyl-L-leucine	0.138	6.5		29	
			24		66	
			24		27	
	L-Leucylglycylglycine	0.0448	5.5	21	71	
			24	91	101	
			2	20	43	
	Diglycylglycine	0.224	5.5	69	94	
			1.5	71		87
			24	107		149
	L-Prolylglycine	0.224	3	16	17	
			6.5	37	39	
			24	81	84	
	Glycyl-L-proline	0.224	3		34	
			6.5		76	
			24		93	
	Glycylglycine	0.224	24	20		49
			48	28		62
	Glycyl-L-phenylalanine	0.224	24	32	71	30
			48	44	84	44
	Benzoylglycine	0.224	48	0		

TABLE IV  
*Peptidases of Human Uterine Tissue*

The extracts were prepared from two different uteri obtained surgically. The enzymatic experiments were performed in veronal buffer at pH 8.0 to 8.2 and 40°.

Substrate	Protein N per cc test solution	Time	Hydrolysis		
			No activation	0.001 M Mn <sup>++</sup>	0.001 M Co <sup>++</sup>
	mg	hrs	per cent	per cent	per cent
L-Leucylglycine	0.066	1.5	21	24	
		4	52	50	
		6	66	68	
L-Leucylglycylglycine	0.066	1.5	43	44	
		2	73	72	
		4	96	100	
Diglycylglycine	0.066	1.5	27		
		2	45		
		4	62		
		6	80		
Glycyl-L-proline	0.164	5	11	14	
		24	36	88	
Glycyl-L-phenylalanine	0.164	3		34	
		5		42	
		24		71	
Benzoylglycine (pH 7.8)	0.246	24	1	0	0
" (" 5.0)	0.246	24	1		
L-Leucinamide	0.132	3	16	37	
		4	31	61	
L-Leucylglycylglycine	0.132	0.75	24	26	
		2	61	65	
Diglycylglycine	0.132	0.75	25	12	
		2	53	34	
		2.5	64	43	
		3	75	52	
		4	94	63	
Glycyl-L-leucine	0.132	0.75	38	10	60*
		2	59	22	100*
		3.5	76	38	
Glycylglycine	0.264	0.75	22	48	74
		1.5	41	77	100
		2.75	58	102	101
Glycyl-L-proline	0.396	3.5		74	
Glycylhydroxy-L-proline	0.396	3.5		14	

\* This solution contained 0.001 M Zn<sup>++</sup>.

and the properties of a leucyl peptidase which was activated by Mg<sup>++</sup>. Other peptides were also hydrolyzed by this extract. Fruton (2) has stud-

red the peptidases of human skin and found that several enzymes were present, including a leucine aminopeptidase activatable by  $Mn^{++}$ , prolidase, and a peptidase which hydrolyzed LGG and GGG

Table IV presents data obtained with aqueous extracts of human uterus. These extracts contain high peptidase activities similar to those already described in the preceding sections. However, the properties of some of the activities are different from those obtained from other species. The hydrolysis of glycyl-L-leucine by extracts of rabbit tissue is activated by  $Mn^{++}$  and  $Co^{++}$ . With the human extracts,  $Mn^{++}$  and  $Co^{++}$  inhibit the hydrolysis of this peptide, but strong activation is produced by  $Zn^{++}$ .

The hydrolysis of glycylglycine is activated by  $Mn^{++}$ , and to a much greater extent by  $Co^{++}$ . This behavior appears to be general for all tissues investigated thus far. As obtained from most tissues, the enzyme is extremely labile. In contrast, the uterine dipeptidase is quite stable at ice box temperatures for many weeks. The properties of these glycylglycine dipeptidases will be described elsewhere (5).

Carboxypeptidase and endopeptidases have not been found in extracts of human uterus when tested under the same conditions described for the other tissues.

#### *Some Observations on Nature of Tissue Peptidases*

*Leucine Aminopeptidase*—Linderström-Lang (11, 12) first pointed out that LG was hydrolyzed by a distinct leucyl peptidase. Berger and Johnson (10, 13) later observed that this enzyme is extremely widely distributed in nature, and that it is strongly activated by  $Mn^{++}$  or  $Mg^{++}$ . It has since been shown that the enzyme fulfils the specificity requirements of an aminopeptidase (1), and that the activation is apparently a true combination of metal and protein (14). It is of some interest to determine which metal is actually present in the naturally occurring enzyme.

Since  $Mg^{++}$  ions form a soluble complex with citrate, whereas  $Mn^{++}$  ions do not, it is possible to differentiate between these two metal ions<sup>1</sup>. Various tissue extracts were studied in the presence and absence of 0.01 M sodium citrate without the addition of metal ions (Table V). The hydrolysis of LG and L-leucinamide (LA) was found to be strongly inhibited by citrate ions. It would appear, therefore, that the naturally occurring leucine aminopeptidases are magnesium enzymes. This finding is similar to the observations of McCarty (15) on citrate inhibition of the desoxyribonuclease of beef pancreas.

*Leucylglycine-Splitting Enzyme of Human Uterus*—Thus far, several different enzymes of animal tissues are known which hydrolyze leucine pep-

<sup>1</sup> This refers to the situation in the neutral pH range. In strongly alkaline solution, citrate will form complexes with  $Mn^{++}$  and many other divalent ions.



tides These include two types of leucine aminopeptidases, the  $Mg^{++}$ - or  $Mn^{++}$ -activated enzymes (1, 2, 10), and the cysteine-activated enzymes of beef spleen, beef kidney, and swine kidney (16) These aminopeptidases

TABLE V  
*Effect of Citrate on Tissue Peptidases*

The tissue extracts were used without the addition of metal ions \* The tests were performed at 40° in veronal buffer at pH 7.8 to 8.0

Tissue	Substrate	Protein N per cc test solution	Time	Hydrolysis	
				No citrate	0.01 M citrate
		mg	hrs	per cent	per cent
Rabbit muscle	L-Leucylglycine	0.50	3	55	30
			4.5	94	45
" " (partially purified)	L-Leucinamide	0.50	2	46	21
			4	83	40
	Glycyl-L-proline	0.25	2	17	17
			3	28	26
			5	47	47
Rabbit heart	L-Leucylglycine	0.126	24	102	100
			1.5	16	5
	L-Leucinamide	0.126	20	102	20
			4	41	11
			6	53	13
" uterus	L-Leucylglycine	0.224	24	101	30
			2	26	11
	L-Prolylglycine	0.224	5	73	27
			3	16	21
			6.5	37	32
	Glycyl-L-proline	0.224	24	81	81
			3	26	26
5			41	40	
Hog intestine (VioBin)	L-Leucylglycine	0.105	24	84	85
			1	30	20
			2	67	27
	L-Leucinamide	0.262	4	99	43
			0.75	59	32
			1.5	99	45

\* The activity of these extracts towards the various substrates is from 10 to 100 times greater in the presence of 0.001 M  $MnCl_2$ .

appear to require similar structural specificity in their substrates, since LG, LGG, and LA are hydrolyzed with velocity constants of the same magnitude. In addition to these enzymes, a peptidase is known which hydrolyzes LGG, GGG, and other tripeptides, this enzyme has little or no action on LG and LA (1)

The experiments reported in Table IV show that uterine extracts contain a tripeptide-splitting enzyme as judged by the hydrolysis of GGG. This hydrolysis is not metal-activated and shows some inhibition by  $0.001 \text{ M Mn}^{++}$ . Part of the hydrolysis of LGG must also be due to this enzyme. A metal-activated leucine aminopeptidase is present, since the hydrolysis of LA is activated by  $\text{Mn}^{++}$ .

It has already been demonstrated that the leucine aminopeptidases are magnesium proteins as judged by the inhibition which is produced by citrate. Table VI shows that in a crude extract the hydrolysis of LG and LA is partly inhibited by citrate. The hydrolysis of LGG by the tripeptide-splitting enzyme is apparently too rapid for the small amount of aminopeptidase to influence the over-all rate of hydrolysis, since there is no activation by  $\text{Mn}^{++}$  or inhibition by citrate.

The hydrolysis of the different substrates by the uterine extract follows the kinetics of a zero order reaction. The proteolytic coefficient,  $C^0$ , was calculated from the zero order velocity constant  $K^0$ , which is expressed as per cent hydrolysis per minute. In Table V, the coefficient  $C^0$  is equal to  $K^0/E$  where  $E$  is the enzyme concentration in mg of protein N per cc of solution.

It was found that the precipitate obtained with 2 volumes of cold acetone, and then washed with cold acetone and dried, gave a highly active solution when redissolved in water. The relative activity towards the different substrates was changed considerably (Table V). With the acetone-treated preparation, a considerable portion of the tripeptide-splitting enzyme was destroyed, since  $C^0$  for GGG decreased from 3.9 to 0.48 and  $C^0$  for LGG from 5.9 to 1.65. Much of the leucine aminopeptidase activity was retained, since an activating effect of  $\text{Mn}^{++}$  was apparent both with LGG and LA. However, the activity towards LG was greatly concentrated, since  $C_{\text{LG}}$  increased about 6-fold from 0.94 to 5.7.

It seems that this activity towards LG must represent a distinct enzyme of a hitherto unknown type. This is suggested by the following evidence. No activation was produced by  $\text{Mn}^{++}$ , and no inhibition occurred in the presence of citrate. The main action on LG cannot be due to leucine aminopeptidase, since activation was produced by  $\text{Mn}^{++}$  when the same preparation was tested with LGG or LA. The activity towards LG was not activated by other metals, such as  $\text{Mg}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Co}^{++}$ , or  $\text{Zn}^{++}$ . Some inhibition was produced by the last two ions. The metal-activated leucine aminopeptidases of hog intestine (1) and of rabbit muscle (unpublished observations) hydrolyze LG and LGG at equal rates. The acetone preparation of uterus hydrolyzed LG about 4 times faster than LGG in the absence of  $\text{Mn}^{++}$ . Since at least part of the splitting of LGG is due to the concurrent action of the tripeptide-splitting enzyme and the aminopepti-

TABLE VI

*Hydrolysis of Leucine Compounds by Crude and Acetone-Treated Extracts of Human Uterus*

The tests were performed at 40° and pH 8.2 in veronal buffer. The proteolytic coefficient,  $C^0$ , was calculated from the zero order velocity constant,  $K^0$ , where  $C^0 = K^0/E$ .  $E$  is expressed as mg of protein N per cc of test solution.

Substrate	Protein N per cc test solution	Time	No addition		Hydrolysis in 0.001 M $Mn^{++}$	Hydrolysis in 0.01 M citrate
			Hydrolysis	$C^0$		

Tests performed with crude extract

	mg	hrs	per cent		per cent	per cent
L-Leucylglycylglycine	0.111	1	41	6.1	36	40
		1.5	58	5.8	50	62
		2	76	5.7	62	76
L-Leucylglycine	0.222	1.5	17	0.86	18	9
		2.5	31	0.95	32	17
		3.5	44	0.95	43	28
		5	67	0.99	58	41
L-Leucinamide	0.222	2	14	0.53	25	8
		4	23	0.43	50	11
		6	35	0.44	73	16
		24	88		102	38
Diglycylglycine	0.111	0.5	12	3.6		
		1	28	4.2		
		1.5	41	4.1		
		2	51	3.8		
Carbobenzoxy-L-leucylglycine	0.222	24	2			
Carbobenzoxy-L-leucinamide	0.222	24	0			

Tests with acetone precipitated preparation

L-Leucylglycylglycine	0.181	1	17	1.6	35	17
		1.5	25	1.5	55	22
		2	36	1.7	76	26
		2.5	49	1.8	83	30
L-Leucylglycine	0.181	0.25	15	5.5	17	16
		0.5	31	5.7	36	30
		0.75	47	5.7	48	46
		1	62	5.7	61	62
		1.5	83		83	84
L-Leucinamide	0.362	0.5	15	1.4	23	15
		1	31	1.5	47	30
		1.5	44	1.4	70	44
		2	60	1.4	85	57
		3	85	1.3	100	86
Diglycylglycine	0.362	1	10	0.46		
		2	21	0.48		
		3	32	0.49		
		4.5	47	0.48		
		5.5	58	0.48		
Carbobenzoxy-L-leucylglycine	0.362	24	1			

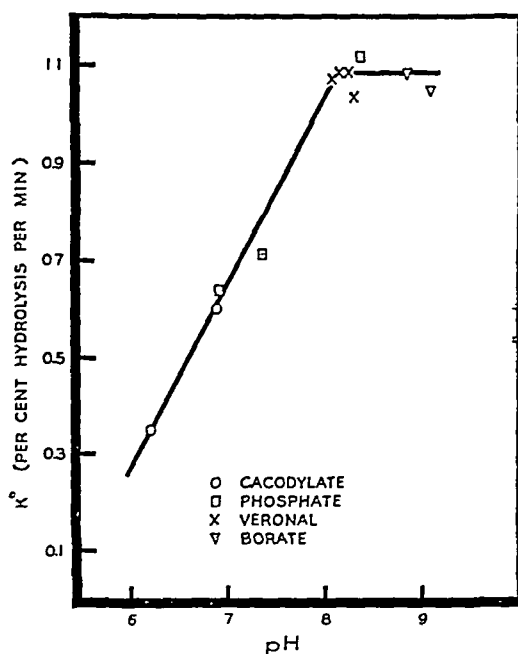


FIG 1 Hydrolysis of L-leucylglycine as a function of pH The activities were computed from the zero order velocity constants The tests were performed at 40° with an acetone-treated enzyme at a concentration of 0.181 mg of protein N per cc of test solution The buffers were at a concentration of 0.04 M, except for the cacodylate which was used at 0.1 M

TABLE VII

*Effect of Inhibitors on Hydrolysis of L-Leucylglycine*

The acetone-treated enzyme was used at a concentration of 0.181 mg of protein N per cc of test solution The experiments were performed at 40° in veronal buffer of pH 8.2

Time <i>min</i>	Hydrolysis				
	No addition <i>per cent</i>	0.1 M fluoride <i>per cent</i>	0.01 M fluoride <i>per cent</i>	0.04 M cyanide <i>per cent</i>	0.003 M cysteine <i>per cent</i>
15	16		8	14	14
30	32	13	18	28	28
45	48	18	25	41	39
60	63	22	34	51	45

dase, the new enzyme must possess a far greater activity towards LG than it does towards LGG

The new enzyme does not seem to be an aminopeptidase, since the acetone-treated preparation showed a greater increase of activity towards LG

than towards LA in the presence or absence of added  $Mn^{++}$ . The presence of a carboxypeptidase can be excluded, since no hydrolysis of carbobenzoxy-L-leucylglycine was observed in 24 hours. It is tentatively suggested that the new enzyme might be a specific dipeptidase. It has already been found that the hydrolysis of glycylglycine by several animal tissues is due to a specific dipeptidase (5), and there is evidence that the splitting of certain other dipeptides may also be due to specific dipeptidases (unpublished observations).

The effect of pH on the hydrolysis of LG by the acetone-treated enzyme is shown in Fig. 1. The enzyme shows a broad zone of maximal activity

TABLE VIII

*Effect of Incubation of Prolidase with  $MnSO_4$*

The enzyme was obtained from a rabbit muscle extract which had been precipitated with acetone and dried. The concentration was 0.248 mg of protein N per cc of test solution. The tests were made at 40° and pH 8.0 in veronal buffer. Incubation of  $Mn^{++}$  and enzyme was at 40° and pH 8.0 for 1 hour prior to addition to the substrate (glycyl-L-proline).

Time  <i>hrs</i>	Hydrolysis		
	No activator	0.001 M $Mn^{++}$ added without incubation	0.001 M $Mn^{++}$ incubated with enzyme
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
0.5		8	49
0.75			62
1		17	67
2	17	40	92
3	28	63	101
5	47	99	
24	102		

extending from pH 8.1 to at least pH 9.1. No specific ion effects were observed.

The effect of some inhibitors on the hydrolysis of LG is given in Table VII. The high concentration of sodium cyanide has only a slight effect. This is probably due to its action on the leucine aminopeptidase which is present, since the hydrolysis of LG by this enzyme is known to be strongly inhibited by cyanide (17, 18). Cysteine appears to have a slight initial inhibitory effect which progressively increases. Fluoride in 0.01 M concentration produces a 50 per cent inhibition, while 0.1 M fluoride causes a 63 per cent inhibition.

*Prolidase*.—The hydrolysis of glycyl-L-proline and glycylhydroxy-L-proline by extracts of intestinal mucosa has been ascribed to a distinct peptidase which does not require the presence of peptide hydrogen. This

enzyme has been found to be activated by  $Mn^{++}$ , but not by other divalent ions (1) In this investigation on other tissues, the prolidases are also specifically activated by  $Mn^{++}$  ions Moreover, no inhibition of activity was observed in the presence of 0.01 M citrate (Table V), so that additional

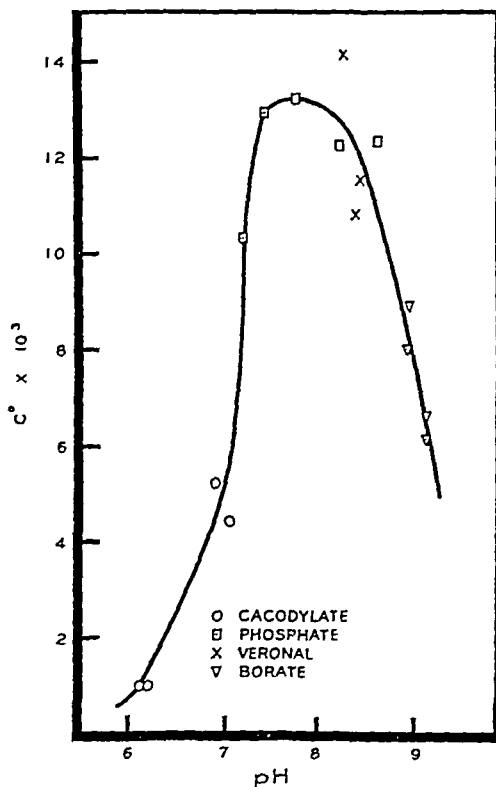


FIG 2 Activity of prolidase as a function of pH  $C^\circ$  is the first order velocity constant per mg of protein N per cc The enzyme was obtained from an aqueous extract of rabbit muscle, this was precipitated with 2 volumes of cold acetone and dried The aqueous extract of the acetone powder was filtered and used for the tests The enzyme concentration was 0.496 mg of protein N per cc of test solution The enzyme was incubated at  $40^\circ$  with 0.001 M  $MnSO_4$  for 2 hours prior to addition to the substrate (glycyl-L-proline) Incubation at pH 8.0 or at the pH of the tests had the same effect on the subsequent rate of hydrolysis of the substrate

evidence is now available that these enzymes are specific metal proteins in contrast to leucine aminopeptidase which can be activated by either  $Mn^{++}$  or  $Mg^{++}$  Likewise, prolinase (hydrolysis of L-prolylglycine), which is activated only by  $Mn^{++}$ , is not affected by citrate (Table V)

The activation of prolidase by  $Mn^{++}$  is a time reaction (Table VIII)

After incubation of  $Mn^{++}$  and the enzyme for 1 hour at  $40^\circ$  and pH 8.0, the hydrolysis proceeds with regular first order kinetics.  $C$ , the first order velocity constant per mg of protein  $N$ , is about 10 times greater in the presence of  $Mn^{++}$  (0.037) than in the absence of metal ions ( $C = 0.0032$ ). This type of time reaction between a metal and its specific protein, which was first described for leucine aminopeptidase in 1941 (19), has since been found for aiginsase (20) and phosphatase (21).

The effect of pH on the activity of the prolidase of rabbit muscle is shown in Fig. 2. The enzyme possesses a broad range of maximal activity between pH 7.5 and 8.2.

Although the specificity of prolidase has not yet been elucidated completely, some information is available. Crude and partially purified ex-

TABLE IX  
*Homospecificity of Prolidases*

The enzyme preparation was incubated at  $40^\circ$  in veronal buffer at pH 8.0 together with 0.01 M  $Mn^{++}$  for 1 to 2 hours prior to addition to the buffered substrate solution. The final test solutions contained 0.002 M  $Mn^{++}$ . The proteolytic coefficient  $C = K/E$  where  $K$  is the first order velocity constant for the enzyme concentration  $E$  expressed in mg of protein  $N$  per cc of test solution.

Enzyme preparation	$C_{GP}$	$C_{GHP}$	Proteolytic quotients, $C_{GP}/C_{GHP}$
Rabbit skeletal muscle (crude)	0.0052	0.00066	7.9
"    "    "    (partially purified)	0.0131	0.00164	8.0
"    heart muscle (crude)	0.0074	0.00088	8.4
Rat skeletal    "    "	0.0056	0.00073	7.7
Human uterus	0.0029	0.00032	9.1
"    serum	0.00064*	0.000088*	7.3

\*  $K$  for 0.5 cc of serum in 2.5 cc of enzyme test solution

tracts of hog intestinal mucosa were found to hydrolyze glycyl-L-proline about 8 times faster than glycylhydroxy-L-proline (1). All of the tissues which were examined in this investigation were found to hydrolyze the hydroxyproline peptide as well as the proline peptide. The data in Table IX summarize the results. From the relative constancy of the ratio  $C_{GP}/C_{GHP}$ , it would appear that these enzymes are homospecific (22). Moreover, the conclusion is probably justified that we are dealing with the activity of a single enzyme from each tissue, since it would be a remarkable coincidence to obtain such constant ratios if more than one enzyme were involved.

Prolidases of intestinal mucosa (1) and of the tissues studied in this investigation do not hydrolyze carbobenzyglycyl-L-proline or carbobenzyglycylhydroxy-L-proline, a free amino group appears to be required for

the action of these enzymes. In preliminary experiments on the purification of rabbit muscle prolidase, it was found that the hydrolysis of glycylglycyl-L-proline paralleled the hydrolysis of diglycylglycine and not that of glycyl-L-proline. It would seem that the imino peptide bond must be adjacent to a free amino group.

#### DISCUSSION

The classical picture of protein digestion has portrayed an intestinal erepsin as concerned with the terminal stages of the breakdown of peptides to free amino acids. In recent years, it has become apparent that this viewpoint is erroneous in two important aspects. First of all, it is now obvious that the peptidases of intestinal mucosa represent an extremely complex mixture of many different enzymes of exceedingly diverse specificity. (1) The term erepsin then becomes a misleading one in so far as it is used to describe a single enzyme or a mixture of a few enzymes. Secondly, evidence has gradually accumulated that the peptidases of intestinal mucosa are not unique. The same or similar enzymes have been found in many other types of animal tissues. Maschmann (7) has found these activities in liver and kidney. Fruton (2) has shown that these enzymes are present in skin, lung, and serum. It has now been demonstrated that similar peptidases are present in skeletal muscle, heart, and uterus. The presence of these enzymes in various tissues throws some doubt on the viewpoint that the enzymes of intestinal mucosa are solely concerned with digestion. It is more likely that all of these enzymes are intracellular in nature and concerned with similar functions, regardless of the tissue in which they are found.

#### EXPERIMENTAL

The enzyme experiments were performed in 25 cc volumetric flasks at a temperature of 40°. Hydrolysis was measured on 0.2 cc samples by the titration method of Grassmann and Heyde (23). The substrates were present in a concentration of 0.05 M. Hydrolysis of one peptide bond is expressed as 100 per cent. Controls were performed by incubation of the tissue extracts in the absence of substrate. No significant autolysis was detected.

The extracts were prepared by washing the tissues with cold water to remove as much blood as possible. The tissues were then minced and homogenized with cold distilled water in a Waring blender. The extracts were adjusted to between pH 7.2 and 7.5 (phenol red) and centrifuged or filtered. No differences in activity were found when the extracts were prepared in physiological saline or veronal buffer (pH 7.8), except that more inactive protein was dissolved.



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#### SUMMARY

1. Extracts of rabbit muscle, heart, and uterus as well as those of rat muscle and human uterus contain high peptidase activities which can hydrolyze a variety of simple peptides. Leucine aminopeptidase, prolidase, and a tripeptide-splitting enzyme have been found in all of these tissues. Endopeptidases that hydrolyze peptide linkages, which are not adjacent to free terminal amino or carboxyl groups, have not been found to any significant extent. Carboxypeptidase has not been detected in these extracts.

2. The metal-activated leucine aminopeptidases of various tissues are strongly inhibited by citrate ions, indicating that these enzymes are magnesium protein compounds.

3. Some evidence is presented for a new type of enzyme in human uterus which hydrolyzes L-leucylglycine, and which has at best a very much weaker action on L-leucylglycylglycine and L-leucinamide. This suggests that an aminopeptidase is not responsible for the action. Blocking of the amino group (carbobenzoxy-L-leucylglycine) prevents the hydrolysis, hence the action of a carboxypeptidase can be excluded. The new enzyme may be a specific dipeptidase.

4. The prolidases of various tissues and human serum are homospesific manganese enzymes which hydrolyze glycyl-L-proline and glycylhydroxy-L-proline with the same relative effectiveness.

5. The concept of an intestinal erepsin is outmoded and misleading, since the same or similar enzymes have been found in many other types of tissues.

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# THE GLYCYLGLYCINE DIPEPTIDASES OF SKELETAL MUSCLE AND HUMAN UTERUS\*

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Until recent years, it was generally assumed that the hydrolysis of all simple dipeptides was due to a single enzyme, dipeptidase. In 1929, Linderström-Lang (1) demonstrated that the two dipeptides, leucylglycine and alanylglycine, were split by different enzymes. One of these has since been found to be a leucine aminopeptidase which hydrolyzes a variety of leucine peptides including leucinamide (2). Because of this and similar studies, the existence of a dipeptidase which is capable of hydrolyzing many kinds of dipeptides has become doubtful. The studies of Bergmann and his collaborators (3, 4), have amply demonstrated that proteolytic enzymes require for their action the presence of specific amino acid residues in the substrates as well as the presence or absence of amino and carboxyl groups.

It has now been found that the enzyme of animal tissues which hydrolyzes glycylglycine appears to fulfil the specificity requirements of a dipeptidase, but in a sense quite different from that in which the term was used earlier. Substitution of amino or carboxyl groups renders the peptide resistant to hydrolysis. Moreover, the activity towards glycylglycine does not appear to parallel the hydrolysis of any other dipeptide which has yet been studied. It appears justifiable to ascribe the enzyme action to a specific glycylglycine dipeptidase.

## *Properties of Glycylglycine Dipeptidase of Rat Muscle*

*Specificity*—Table I presents the data obtained with a crude fresh aqueous extract of rat skeletal muscle. The splitting of glycylglycine (GG) is strongly activated by  $\text{Co}^{++}$  (5) and to a lesser degree by  $\text{Mn}^{++}$ .  $\text{Mg}^{++}$  does not accelerate this hydrolysis, and  $\text{Zn}^{++}$  acts as an inhibitor. These extracts do not hydrolyze compounds in which the free amino group is suppressed as in carbobenzoxyglycylglycine or benzoylglycylglycine, or in which both the amino and carboxyl groups are substituted as in carbobenzoxyglycylglycinamide and benzoylglycinamide. No hydrolysis of hippuric acid (benzoylglycine) was detected in these extracts, hippuricase is obviously a different enzyme from the dipeptidase.

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The hydrolysis of diglycylglycine (GGG) by crude tissue extracts is due to a distinct enzyme from the dipeptidase. This is shown by the fact that the initial hydrolysis of the tripeptide is only slightly activated by metal ions, and that, in the absence of metal ions, the second peptide bond is split extremely slowly. In the presence of  $Mn^{++}$  or  $Co^{++}$ , the second peptide bond is hydrolyzed quite rapidly (Table II). The tripeptide-splitting enzyme is much more stable than the dipeptidase. Under conditions in which most of the dipeptidase activity is lost, *e.g.* standing at pH 7.6 and  $40^\circ$  for 5 hours, the hydrolysis of the tripeptide is relatively unimpaired. Under the same conditions, most of the capacity of the extract to hydrolyze glycylglycinamide and glycynamide is unaffected. However, since there is

TABLE I

*Action of Rat Skeletal Muscle Extracts on Glycine Compounds*

The tests were performed at  $40^\circ$  in veronal buffer at pH 8.0. The enzyme preparation was a freshly prepared crude extract of rat skeletal muscle.

Substrate	Protein N per cc test solution	Time	Hydrolysis		
			No activator	0.001 M $Mn^{++}$	0.001 M $Co^{++}$
	mg	hrs	per cent	per cent	per cent
Glycylglycine	0.158	3	19	37	83
		24	34	96	102
Benzoylglycinamide	0.316	22	1		0
Benzoylglycine	0.316	22	0	0	2
Carbobenzoylglycylglycine	0.316	22	0		0
Benzoylglycylglycine	0.474	45	4		1
Glycinamide	0.474	22	3		8
Glycylglycinamide	0.316	22	20		20
Carbobenzoylglycylglycinamide	0.316	22			5

some loss of activity towards these substrates, it would appear that the dipeptidase may be able to split the two amides extremely slowly.

The hydrolysis of GGG, L-leucylglycylglycine, and other tripeptides has been ascribed to a distinct enzyme. Although this enzyme has not yet been obtained in homogeneous form, highly active preparations from intestinal mucosa possess only slight activity towards most dipeptides (2).

The enzyme which hydrolyzes GGG may be activated to some extent by  $Co^{++}$ . The enzyme preparations which had been heated at  $40^\circ$  for 5 or 12 hours show little hydrolysis of glycylglycine in 4 hours, yet there is a greater effect of  $Co^{++}$  on the splitting of the tripeptide than can be due to the action of the dipeptidase.

Other glycine-containing peptides are hydrolyzed by a crude extract of rat muscle (6), but these appear to be unrelated to the hydrolysis of GG

Since further studies are in progress on some of these enzymes, only a few salient observations which differentiate some of these activities from GG dipeptidase will be given. Unlike the GG-splitting enzyme, which is quite

TABLE II

*Differentiation of Glycylglycine Dipeptidase from Tripeptide Splitting Enzyme*

The tests were performed at 40° in veronal buffer at pH 7.8 with a fresh extract of rat muscle. The second series was performed with the same extract after it had been incubated at 40° for 5 hours and filtered. The third series was made with the same extract after heating for 12 hours at 40°.

Substrate	Protein N per cc test solution	Time	Hydrolysis		
			No acti- vator	0.001 M Mn <sup>++</sup>	0.001 M Co <sup>++</sup>
	mg	hrs	per cent	per cent	per cent
Glycylglycine	0.185	1	3	17	31
		2	2	28	62
		4	7	51	100
Diglycylglycine	0.185	1	15	16	20
		2	31	25	49
		4	55	47	107
		20	102	136	191
Glycylglycinamide	0.370	24	24	25	24
Glycinamide	0.370	24	11	6	15
Tests with same enzyme after 5 hrs at 40°					
Glycylglycine	0.107	4	2	3	3
		24	11	12	23
Diglycylglycine	0.107	2	26	17	36
		4	46	37	66
		6	64	53	87
		24	102	105	113
Glycylglycinamide	0.214	24	12	12	11
Glycinamide	0.214	24	6	5	8
Tests with same preparation after 12 hrs at 40°					
Glycylglycine	0.079	4	1	1	5
		24	8	10	19
Diglycylglycine	0.158	2	21	18	33
		4	40	33	67
		5.5	54	48	79
		24	98	96	99

labile, the enzyme in rat muscle which hydrolyzes glycyl-L-leucine is much more stable at ice box temperatures. Moreover, this enzyme is not activated by Co<sup>++</sup>. The hydrolysis of glycyl-L-phenylalanine is not acti-

vated by  $\text{Co}^{++}$ , but is activated by  $\text{Mn}^{++}$  and to a much greater degree by  $\text{Mg}^{++}$ . The enzyme which hydrolyzes glycyl-L-alanine is not affected by  $\text{Co}^{++}$ . Leucine aminopeptidase, prolidase, and prolinase have all been obtained free of the GG dipeptidase.

*Product of Hydrolysis*—Since the action of the dipeptidase appears to be so specific, it was desirable to isolate the compound formed after the action of the tissue extracts. The expected amount of glycine was isolated as the carbobenzoxy compound. The details are given in the experimental section.

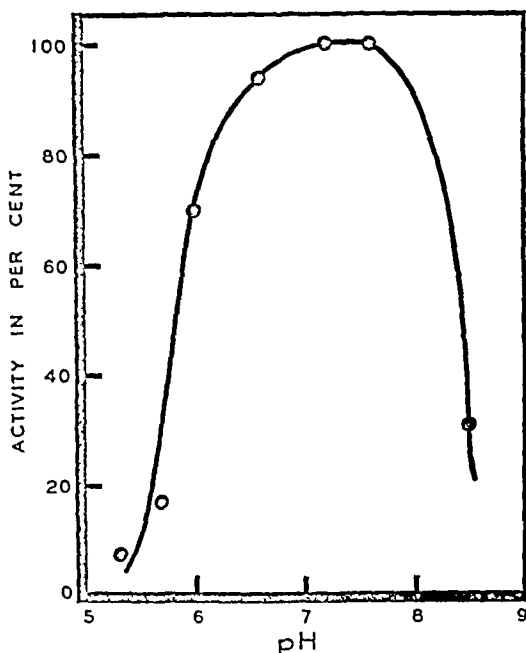


FIG 1 Stability of glycylglycine dipeptidase as a function of pH as determined after incubation at  $40^\circ$  for 2.5 hours. Residual activity of the enzyme was then determined in veronal buffer at pH 8.0 in the presence of  $0.001\text{ M Co}^{++}$ . The enzyme preparation was a crude extract of rat muscle.

*Stability*—Fig 1 shows the results of an experiment in which a fresh extract of rat skeletal muscle was adjusted to a series of different pH values with requisite amounts of  $0.1\text{ M NaOH}$  or  $\text{HCl}$ . After incubation at  $40^\circ$  for 2.5 hours, the solutions were readjusted to pH 8.0. The specific enzyme activities as determined from the zero order velocity constants are plotted in Fig 1 as a percentage of the maximal activity which was observed.

The dipeptidase is obviously an extremely labile enzyme and even at the region of maximal stability, about pH 7.5, about 50 per cent of its activity may be lost on standing at ice box temperatures overnight. The presence of  $\text{Co}^{++}$  or  $\text{Mn}^{++}$  in optimal amounts ( $0.001\text{ M}$ ) improves the stability somewhat.

*Kinetics, Effect of Enzyme, pH, and Cobalt Concentration*—The hydrolysis of GG follows zero order kinetics under the conditions which we have used, provided that the measurements are made within a few hours so that the inactivation of the enzyme may be considered to be negligible. In fact, the enzyme is greatly stabilized by the presence of its substrate. Fig 2 shows that the amount of hydrolysis is proportional to the enzyme concentration over a reasonable range. The values obtained at the two lowest enzyme

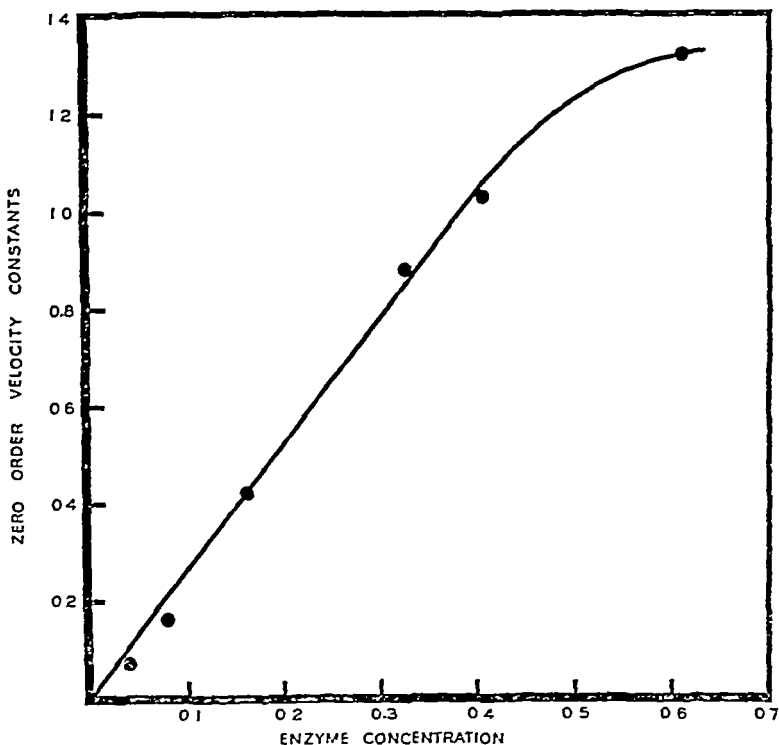


FIG 2 Activity as a function of enzyme concentration (in mg of protein N per cc) for an extract of rat skeletal muscle. The zero order velocity constants are in per cent hydrolysis per minute. The experiments were performed at  $40^{\circ}$  in veronal buffer at pH 8.0 in the presence of  $0.001\text{ M Co}^{++}$ .

concentrations are somewhat low. This is probably due to a partial inactivation of the enzyme over the period of 5 to 6 hours necessary for adequate measurements of hydrolysis. At the higher enzyme concentrations, satisfactory velocity constants could be obtained within 1 to 2 hours.

The presence of  $0.001\text{ M Co}^{++}$  ions is sufficient to produce maximal activation of the enzyme. Because of the lability of the enzyme, the effect of different  $\text{Co}^{++}$  concentrations has to be determined during a short time



with a high enzyme concentration. The data are shown in Fig 3 where the zero order velocity constants are presented as a function of  $\log \text{Co}^{++}$  concentration.  $K_d$ , the dissociation constant of a hypothetical enzyme-metal compound, is equal to  $2.8 \times 10^{-5} \text{ M}$ . The velocity constant obtained without the addition of metal ions is also indicated in Fig 3. The maximal activation produced was 10 times that found in the absence of  $\text{Co}^{++}$ .

*Effect of Cysteine*—When metal ions are not added to the tissue extracts, the hydrolysis of GG is strongly inhibited by the presence of 0.003 M cysteine. This inhibition, which can be partly reversed by the addition of  $\text{Mn}^{++}$  or  $\text{Co}^{++}$ , may be interpreted as a binding of metal ions by cysteine in

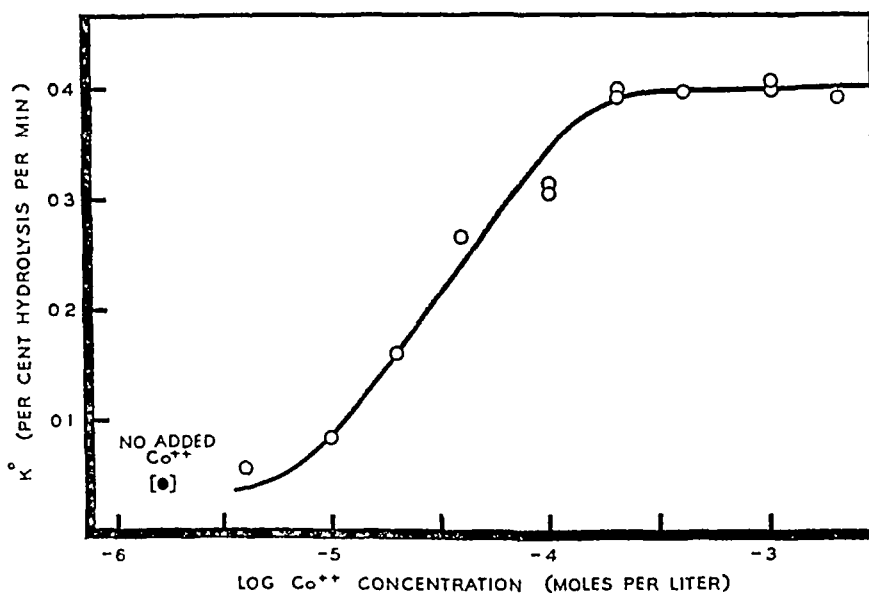


FIG 3 Activity (zero order velocity constants,  $K^0$ ) as a function of  $\text{Co}^{++}$  concentration for the splitting of glycylglycine by rat muscle. The solid point represents the activity in the absence of added metal ions.  $K_d$ , the dissociation constant, is  $2.8 \times 10^{-5} \text{ M}$ .

competition with the protein. The formation of cysteine complexes with cobalt and other heavy metals is well known (7). The GG dipeptidase is not related to the cysteine-activated cathepsins or to the bacterial enzymes whose activity is enhanced by the presence of both cysteine and metal ions (Maschmann (8)).

#### *Glycylglycine Dipeptidase of Human Uterus and Other Tissues*

The splitting of GG is performed by many types of tissues, and, in all cases, is strongly activated by  $\text{Co}^{++}$  (5, 9). Maschmann (5) used, among other sources, liver, kidney, and intestinal mucosa of rabbit, guinea pig, and

mouse. These findings suggest that the specific dipeptidase is widely distributed in tissues. However, no specificity studies seem to have been performed.

Human uterine tissue has proved to be an extremely rich source of peptidases, and particularly of the GG-splitting enzyme (6). In contrast to

TABLE III  
*Hydrolysis of Glycine Compounds by Tissue Extracts*

These experiments were performed at 40° in veronal buffer at pH 8.0

Tissue	Substrate	Protein N per cc. test solution	Time	Hydrolysis		
				No acti- vator	0.001 M Mn <sup>++</sup>	0.001 M Co <sup>++</sup>
		mg	hrs	per cent	per cent	per cent
Human uterus	Glycylglycine	0.264	0.75	22	54	70
			2	55	98	100
	Diglycylglycine	0.132	0.75	25	12	24
			2	53	34	100
			3	75	52	
			4	98	63	
	Benzoylglycylglycine	0.264	24	0		0
	Benzoylglycine	0.264	24	1		
	Benzoylglycinamide	0.264	24	1		0
	Glycinamide	0.264	24	6		6
	Glycylglycinamide	0.264	24	14		10
	Carbobenzoxylglycylglycinamide	0.264	24	2		1
Rabbit skeletal muscle	Glycylglycine	0.251	2	5	18	15
			24	15	86	82
	Diglycylglycine	0.251	2	20	36	
Rabbit uterus	Glycylglycine	0.224	24	81	145	
			24	20		49
			2	2		19
" heart muscle	"	0.063	24	7		77
			24	7		77
Rabbit serum	"	0.2 cc. in	7	8		22
		0.5 cc.	24	14		52
Hog intestine	"	0.80	1	12		28
			4	37		103
Human serum	"	0.5 cc. in	48	18	46	80
		2.5 cc.				

the enzyme found in most tissues, the uterine enzyme was found to be stable for many weeks at ice box temperatures, and this proved to be an extremely useful property.

Table III shows the data obtained on the hydrolysis of various glycine-containing compounds. The specificity appears to be identical with the

homologous enzyme of rat muscle. It should be noted that  $Mn^{++}$  inhibits the splitting of GGG. The initial rate of hydrolysis of the tripeptide is not accelerated by  $Co^{++}$ , but the later hydrolysis is markedly increased. As in the case of the enzyme from rat muscle, the hydrolysis of the first peptide

TABLE IV

*Effect of Enzyme Concentration on Glycylglycine Hydrolysis*

The enzyme preparation was an aqueous extract of acetone-dried powder from human uterus. The tests were performed in the presence of 0.001 M  $CoCl_2$  at 40°. The solutions were buffered at pH 8.1 with veronal.  $K^0$  is given as per cent hydrolysis per minute.  $C^0$  is  $K^0$  per mg. of protein N per cc.

Protein N	Time	Hydrolysis	$K^0$	$C^0$	$C^0$ , average
mg. per cc.	hrs.	per cent			
0.00452	4	15	0.063	13.9	13.4
	5	17	0.057	12.6	
	6	22	0.061	13.5	
	7	26	0.062	13.7	
0.00904	3.5	24	0.114	12.6	13.2
	4.5	33	0.122	13.5	
	5.5	39	0.118	13.1	
	6	45	0.125	13.8	
	7	50	0.119	13.2	
0.0181	1.5	22	0.244	13.5	13.3
	2	29	0.242	13.4	
	2.5	36	0.240	13.3	
	3	42	0.233	12.9	
0.0362	1	29	0.48	13.3	13.3
	1.5	45	0.50	13.8	
	2	60	0.50	13.8	
	2.5	71	0.47	13.0	
	3	83	0.46	12.7	
0.0724	0.25	14	0.93	12.8	13.6
	0.5	28	0.93	12.8	
	0.75	50	1.11	15.6	
	1	59	0.98	13.5	
	1.25	73	0.97	13.4	
0.109	0.25	23	1.53	14.0	14.0
	0.5	49	1.63	15.0	
	0.75	69	1.53	14.0	
	1	84	1.40	12.8	

bond of the tripeptide is due to a distinct enzyme. In the uterine extracts, the  $Co^{++}$ -activated dipeptidase is more active than the tripeptide-splitting enzyme. It is obvious that as fast as GG is liberated from the tripeptide it will be split by the dipeptidase and thus produce an apparent activation by

$\text{Co}^{++}$  The splitting of glycynamide and glycyglycynamide is not accelerated by  $\text{Co}$  ions

Table III also shows the activating effect of  $\text{Co}^{++}$  on the hydrolysis of GG by several other tissues and by human and rabbit sera. Extracts of rabbit skeletal muscle showed the same specificity with various glycine derivatives as that already presented for rat muscle and human uterus.

*Kinetics of Uterine Enzyme*—The hydrolysis of GG by the uterine dipeptidase is proportional to the enzyme concentration over a tested range of 1 to 24 times (Table IV), and the hydrolysis follows zero order kinetics. The experiments were performed by the addition of enzyme to the solution containing the buffered substrate in the presence of 0.001 M  $\text{Co}^{++}$ . No prior

TABLE V

*Metal Activation and Inhibition of Glycylglycine Splitting by Human Uterine Extract*

Each test sample contained 0.264 mg of protein N per cc of the crude extract. The tests were performed at 40° in veronal buffer at pH 8.0. The metal ions were present in 0.001 M concentration.

Substance tested	Hydrolysis			
	45 min	90 min	120 min	165 min
	per cent	per cent	per cent	per cent
$\text{Co}^{++}$	74	100	101	
$\text{Mn}^{++}$	54	77	98	104
$\text{Mg}^{++}$	21	43	55	64
$\text{Zn}^{++}$	4	6	7	10
None	22	41	55	69
Cysteine (0.03 M)	10	20	29	45
“ + $\text{Co}^{++}$	24	43	54	64
“ + $\text{Mn}^{++}$	17	35	52	77
Citrate (0.01 M)	26	47	59	73

incubation of enzyme and metal was found to be necessary, as the enzyme showed its full activity instantaneously. This is in contrast to the behavior of leucine aminopeptidase (2) and prolidase (6) in which the reaction of enzyme and metal takes considerable time.

The precipitate obtained by adding 2 volumes of cold acetone to a crude filtered extract of uterus could be collected and washed with acetone. A filtered aqueous extract of the acetone-dried powder was about twice as active per mg of protein N as the original extract. The experiments in Table IV were performed with an aqueous extract of the acetone powder.

The acetone-dried powder has been found to be a rich source of many of the peptidase activities of human uterus, and these preparations promise to be extremely useful in purification studies. About 80 per cent of the ac-

tivity towards GGG is destroyed by the acetone treatment, while the dipeptidase activity is concentrated about 2-fold. This furnishes additional proof that the dipeptide and tripeptide are hydrolyzed by distinct enzymes.

*pH Activity Function of Uterine Dipeptidase*—In Fig. 4 is shown the activity of the GG dipeptidase as a function of pH. The enzyme is active over a narrow range and shows a sharp maximum near pH 7.6. The tests

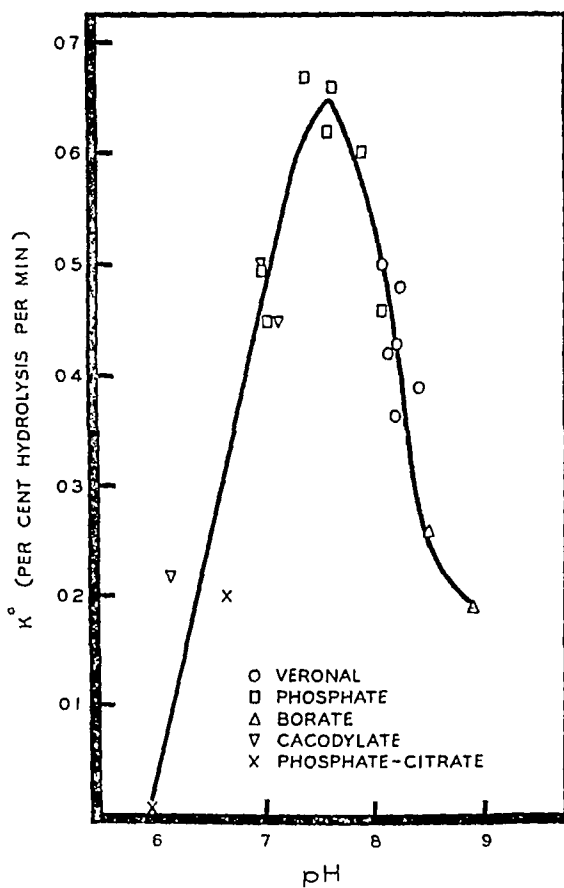


FIG. 4. Activity as a function of pH of the glycylglycine dipeptidase of human uterus. The buffers used were veronal (0.04 M), phosphate (0.04 M), borate (0.04 M), phosphate citrate (0.08 M), and cacodylate (0.1 M). The temperature was 40°. The enzyme was an acetone-precipitated preparation, and was used at a concentration of 0.0362 mg of protein N per cc of test solution.  $\text{Co}^{++}$  was present at 0.001 M.

were performed with the acetone-precipitated enzyme. Hydrolysis was followed until 50 to 60 per cent splitting had occurred. The pH was then determined and found to differ by less than 0.1 unit from the initial values. The pH measurements were made with a glass electrode at room temperature.

*Effect of Metals, Cysteine, and Citrate*—In Table V are presented the data

showing the effect of various metals ions on the hydrolysis of GG by human uterus.  $Mg^{++}$  is without any action, and  $Zn^{++}$  is a powerful inhibitor of the enzyme. Cysteine is also an inhibitor, but this effect may be prevented by  $Co^{++}$  or  $Mn^{++}$ . Citrate is without action on the enzyme.

#### DISCUSSION

It is difficult to relate our study of the GG dipeptidase to many of the earlier investigations on "dipeptidase" because of the variety of peptides which were then used and which were assumed to be hydrolyzed by one and the same enzyme from many different tissues (10). The data presented in this paper strongly suggest that GG is hydrolyzed by an extremely specific dipeptidase which has little or no action on derivatives of the dipeptide in which the amino or carboxyl groups are substituted. It is also well known from earlier observations (11, 12) that glycylsarcosine is not hydrolyzed by crude extracts of tissues which act on GG. The peptide hydrogen is, therefore, essential for the action of the dipeptidase. The enzyme would appear to require three points of attachment to its substrate through the amino group, the carboxyl group, and the peptide hydrogen.

While the nature of the enzyme-substrate combination is unknown, one observation is suggestive in this regard. When glycylglycine is incubated with  $Co^{++}$  ions at pH 8.0, a pink color develops which is much stronger than that given by  $Co^{++}$  alone. Fig. 5 shows the absorption spectrum of 0.01 M  $CoCl_2$  at pH 7.8 in the presence of 0.125 M GG, GGG, and glycine after the mixtures had been allowed to stand for 24 hours at room temperature. The simplest interpretation of this phenomenon is that a specific coordination compound is formed at this pH. The tendency of  $Co^{++}$  to form complex amino compounds is, of course, well known.

The specific combination of GG and  $Co^{++}$  ions leads to the idea that the function of the metal is to act as a bridge in forming the enzyme-substrate compound. The specificity of the enzyme would depend, therefore, not only on the protein, but also on the ability of the metal ion to combine with the substrate. Further studies are now in progress on specific coordination compounds of peptides with metal ions.

#### EXPERIMENTAL

The crude extracts were prepared by homogenizing the tissues in a Waring blender. The preparation was adjusted to pH 7.5 (phenol red) and the insoluble residue removed by centrifugation or filtration. Since the dipeptidase of rat muscle was unstable, the extracts were prepared in the cold and used within 1 to 2 hours after the death of the animal. The observations on extracts of human uterine tissue were generally made within a few days.

Hydrolysis was measured on 0.2 cc samples by the method of Grassmann and Heyde (13). The enzyme experiments were performed in 25 cc volumetric flasks and at a substrate concentration of 0.05 M. Hydrolysis is expressed as 100 per cent for the complete splitting of one peptide bond. Appropriate controls were performed by incubation of the tissue extracts or substrates under the conditions used for the enzyme experiments.

The substrates used in this investigation were prepared as described in the bibliographic citations: glycylglycine (14), diglycylglycine (14), benzoylglycylglycine (14), glycylglycinamide hydrochloride (15), benzoylglycinamide

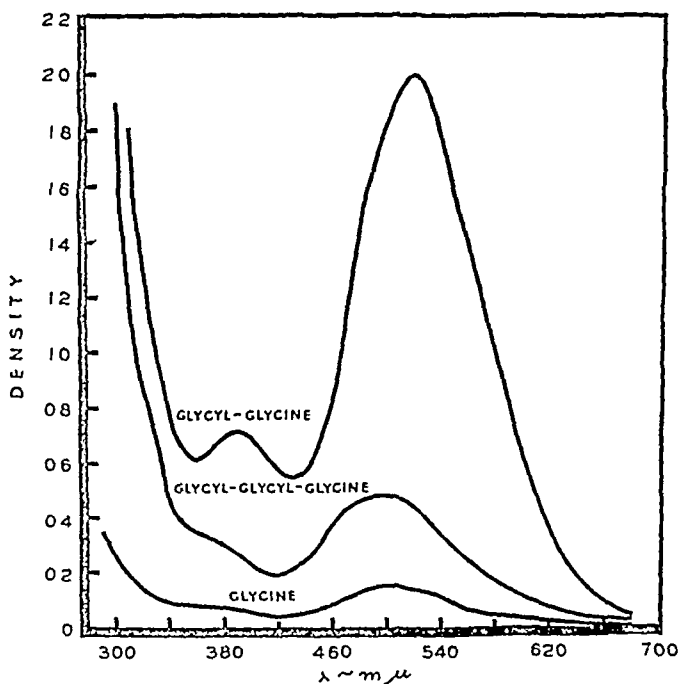


FIG 5 Optical density of  $\text{CoCl}_2$  (0.01 M) in the presence of glycylglycine, diglycylglycine, and glycine at a concentration of 0.125 M. The reaction was allowed to proceed for 24 hours at room temperature (about  $30^\circ$ ). The measurements were made with a Beckman ultraviolet spectrophotometer and a 1 cm quartz cell.

(15), glycylglycinamide acetate (16), carbobenzoxyglycylglycine (17), and carbobenzoxyglycylglycinamide (16). Analytical purity was checked by nitrogen content and by melting point when applicable.

*Isolation of Carbobenzoxyglycine from Hydrolysis of Glycylglycine*—330 mg of glycylglycine were dissolved in water and brought to pH 8 by the addition of NaOH. For the total volume of 50 cc, an extract of rat skeletal muscle was present at a concentration of 0.28 mg of protein N per cc, and  $\text{Co}^{++}$  ions at 0.001 M. When the titration samples showed that 100 per cent splitting had occurred, 1 cc of concentrated HCl was added, and the

mixture was heated in a boiling water bath for 15 minutes. The solution was cooled, filtered with the aid of analytical Celite, and concentrated *in vacuo* to a volume of about 10 cc. The solution was made alkaline to litmus, and 0.8 cc. of carbobenzoyl chloride was added at 0°. On acidification to Congo red, 620 mg. of prisms were obtained. After recrystallization from chloroform the melting point was 120–121°. The mixed melting point with carbobenzoylglycine was 120–121°.

$C_{10}H_{11}O_4N$  (209.2)    Calculated, N 6.70, found, N 6.83

The technical assistance of Rosalind Pack and Marie S. Hanson is gratefully acknowledged.

#### SUMMARY

1. The hydrolysis of glycylglycine by animal tissue extracts is due to a specific dipeptidase which is strongly activated by  $Co^{++}$  and to a lesser extent by  $Mn^{++}$  ions. Hydrolysis may be prevented by substitution of the amino group (benzoylglycylglycine), the carboxyl group (glycylglycinamide), or both (carbobenzoylglycylglycinamide).

2. The glycylglycine dipeptidase of rat muscle is extremely labile and shows maximal stability at pH 7.2 to 7.6. The apparent dissociation constant of the cobalt-enzyme compound is  $2.8 \times 10^{-5} M$ . Hydrolysis follows zero order kinetics under the conditions used.

3. The enzyme is also present in extracts of rabbit skeletal, heart, and uterine tissue and in human uterus. The enzyme of human uterus is stable at low temperatures and it may be precipitated with acetone and dried. The hydrolysis of glycylglycine by this enzyme also follows zero order kinetics and its activity is proportional to the concentration over a wide range. Its maximal activity is at pH 7.6.

4. Glycylglycine forms a specific coordination compound with  $Co^{++}$  as is shown by an intensification of the  $Co^{++}$  spectrum. It is suggested that the formation of such a compound may be significant for the specificity of the enzyme.

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# THE CHARACTERIZATION OF PURINES AND PYRIMIDINES BY THE METHOD OF COUNTER-CURRENT DISTRIBUTION\*

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Adequate criteria are not available for precise characterization of purines and pyrimidines. These compounds do not possess melting points suitable for precise characterization, do not readily form simple derivatives except salts, and nitrogen analyses are never adequate to detect the presence of small amounts of similar impurities. Even their characteristic ultraviolet absorption spectra are generally unsatisfactory owing to similarities of spectra and difficulties in the detection of small contaminations of one by another. Recently Vischer and Chargaff (1) have begun to apply unidimensional paper chromatography to the qualitative characterization of very small samples of adenine, guanine, and xanthine.

The admirable counter-current distribution technique developed by Craig (2, 3) has been applied to this problem and has proved to be a convenient and precise method for qualitative and quantitative characterization of individual compounds and simple mixtures. Estimation of the substances present in the series of tubes resulting from the distribution may be readily made by measurement of the partial absorption spectra. The partition coefficients ( $K$ ) found for a series of purines and pyrimidines and related compounds determined in a system consisting of mutually saturated *n*-butanol and 1 M potassium phosphate buffer of pH 6.5 are given in Tables I and II.

The conditions described are particularly advantageous for the separation of adenine and guanine. The homogeneities (4) of samples of these purines, isolated in the course of certain metabolic experiments (5), have been demonstrated to within 1 or 2 per cent. The presence of small amounts of adenine in commercial samples of hypoxanthine and of guanine in commercial adenine has been readily demonstrated, and routine characterizations have been made of compounds and mixtures encountered in the course of certain synthetic work.

Although the separation of mixtures of the nucleotides would require an impractical number of transfers, they are readily separated from the nucleosides and from the free bases, and thus offers an excellent means for rapid

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analysis of the products of hydrolysis of the nucleotides For instance, the hydrolysis of cytidylic acid in 6 N HCl at 135° for 1 hour yields a mixture of unchanged cytidylic acid, cytidine, and cytosine

TABLE I  
*Partition Coefficients in n-Butanol and 1 M Phosphate, pH 6.5, System*

		Partition coeffi- cient*	Concentration in Tube 0	Absorption maxima
			mg per cc	mμ
Purines	Adenine	2.77†	2.65	260
		2.14†	0.12	
	Guanine	0.45	0.11	247, 274
	Hypoxanthine	0.54	2.00	250
	Xanthine	0.46	0.15	269
	Isoguanine‡	0.28	0.56	239, 286
	Uric acid	0.11	Saturated	240, 293
	2-Thioadenine‡	0.48	0.8	228, 256-262, shoulder at 283
	2,6-Diaminopurine‡	1.21	0.46	250, 281
Pyrimidines	Thymine	1.11	2.00	265
	Uracil	0.401	2.00	260
		0.400	0.56	
	Cytosine	0.207	2.00	267
		0.206	0.50	
	Uramil	0.029	Saturated	End-absorp- tion
	Barbituric acid	0.069	"	275
	4,5,6-Triaminopyrimidine	0.490	0.25	278
	4,6-Diamino-5-formamido- pyrimidine	0.16§	0.30	260
	4,6-Diamino-5-thioformami- dopyrimidine	0.96	0.5	260
	4,6-Diamino-5-benzeneazo- pyrimidine	6.12	0.16	246
	2,4,5,6-Tetraminopyrimidine	0.048	0.48	275

\* Calculated from twelve or twenty-four transfer distributions

† Variations in the constant for adenine are not directly proportional to concentration but are perhaps associated with the purity of the butanol utilized

‡ Prepared by Dr. Aaron Bendich

§ This figure was erroneously given (5) as 0.225

Under conditions that are the basis for a quantitative estimation of purines (6) (1 N HCl at 100° for 1 hour) adenylic acid was demonstrated to be completely hydrolyzed to adenine. Under these conditions the hy-

hydrolysis of yeast (pentose) nucleic acid yielded only the purines, adenine (concentration maximum in Tube 18 out of twenty-four) and guanine (Tube 7), and pyrimidine nucleotides (Tubes 0 and 1). Thymus (desoxypentose) nucleic acid led to a similar distribution pattern with the addition of a trace of material of  $K = 1.0 \pm 0.1$ , probably thymine. The materials in Tubes 0 and 1 in this distribution (apparent  $K = 0.014$ ,  $\lambda_{\max} = 267$ ) were undoubtedly the pyrimidine desoxyribotides, individual samples of which were not available for determination of constants. The work with paper chromatography (1) has also detected no purines other than adenine and guanine in nucleic acid hydrolysates. The more vigorous hydrolysis conditions necessary to split the pyrimidine nucleotides have been shown to result in extensive decomposition of the free purines, and application of

TABLE II

		Partition coefficient
Nucleosides	Adenosine	0.76
	Guanosine	0.12
	Inosine	0.12
	Xanthosine	0.05
	Uridine	0.12
Nucleotides	Cytidine	0.08
	Adenylic acid	0.02 <sub>1</sub>
	Guanylic "	0.02 <sub>0</sub>
	Uridylic "	0.01 <sub>5</sub>
Miscellaneous	Cytidylic "	0.01 <sub>1</sub>
	Picric acid	9.2
	Brucine	1.3

the technique for further characterization of nucleic acids must await studies of the hydrolysis of nucleic acids and their components.

## EXPERIMENTAL

Approximately 0.2 to 2.0 mg per cc of the compound or mixture to be distributed was dissolved in butanol-saturated 1 M phosphate buffer of pH 6.50, or if the mixture were a solution, the pH was adjusted to 6.5 and the buffer concentration was adjusted to approximately 1 M. 8 cc of the solution were introduced into the first compartment (Tube 0) of a twenty-five compartment distribution machine (2). The distribution (2) was usually carried to twenty-four transfers, although in the case of mixtures of adenine and guanine twelve transfers are sufficient, while in the case of uracil and cytosine 56 transfers are needed to obtain a reliable characterization of both components. Typical distribution curves are illustrated in Figs 1

and 2 When the distribution was completed, the contents of the compartments were siphoned into separate test-tubes. From each of the tubes a homogeneous mixture of 1 cc of each phase and 3 cc of 45 per cent aqueous ethanol was prepared. With initial concentrations in the range of 0.2 to 1.0 mg per cc this dilution gives appropriate concentrations for direct measurement of the optical density in a 1 cm cell in the Beckman model

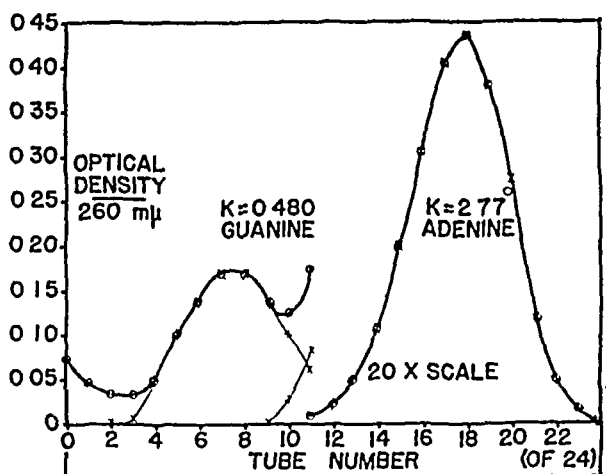


FIG 1 Commercial adenine (initial concentration 2.65 mg per cc) containing about 6 per cent of guanine, twenty-four transfers. Observed points O, calculated X

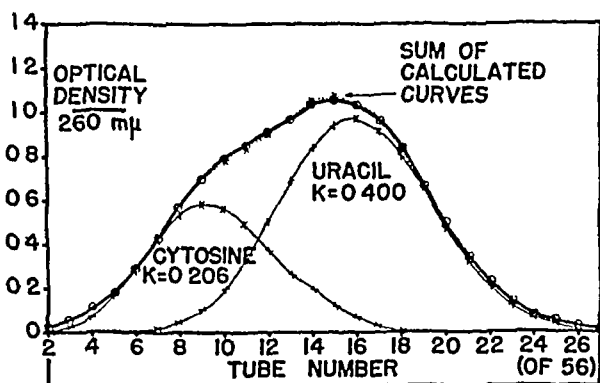


FIG 2 Cytosine (0.20 mg per cc) and uracil (0.20 mg per cc), 56 transfers. Observed points O, calculated X

DU spectrophotometer. If the solutions are more concentrated, larger volumes of ethanol may be used for the dilutions.

The optical densities at 260 mμ, or some other more appropriate wavelength, of each of the solutions were measured and were plotted against tube number, giving the distribution pattern. Calculation of the partition coefficients and construction of the theoretical curves for estimation of

homogeneities were made according to Williamson and Craig (4)<sup>1</sup> With the exception of that of adenine, the partition coefficients have been reproducible within  $\pm 2$  per cent Complete absorption spectra were determined on selected tubes for further qualitative characterization

The components of certain mixtures not readily separated by the solvent pair used, for instance mixtures of guanine with hypoxanthine or with uracil, have been determined by a spectrographic method The extinction coefficients at 264.5  $m\mu$  of guanine and hypoxanthine are equal, and at 256.5  $m\mu$  those of guanine and uracil are equal The absorption at these wave-lengths permits calculation of the total concentration of either of these pairs of substances The values of the extinction coefficients of artificial mixtures of guanine with hypoxanthine and of guanine with uracil were determined at 285  $m\mu$  and plotted against the mole per cent of guanine From the straight line graph thus obtained the mole per cent of guanine may be determined

Difficulty is encountered in getting sufficient pure guanine into solution, particularly in buffers of high salt concentration, but in mixtures with other purines or pyrimidines, guanine is somewhat more soluble The buffered solvent pair described facilitates the analysis of solutions or impure solids since pH adjustments may be readily made For preparative distributions, it has been advantageous to use only butanol and water, in which the partition coefficients have approximately the same values, and from which resolutions may be more easily accomplished The use of a solvent pair consisting of *n*-butanol and water with 0.1 per cent of benzyltrimethylammonium hydroxide and 28 per cent of isopropanol appears to offer certain advantages and is being further investigated

#### SUMMARY

The counter-current distribution technique has been applied to purines and pyrimidines and their derivatives as a precise method of both characterization and determination of homogeneity

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<sup>1</sup> We wish to express appreciation to these authors for the loan of their manuscript prior to its publication



## CRYSTALLINE ALDOLASE\*

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Aldolase, the enzyme that catalyzes the reaction, fructose-1,6-diphosphate  $\rightleftharpoons$  D-glyceraldehyde phosphate + dihydroxyacetone phosphate, was described and investigated in detail by Meyerhof and Lohmann (2). Herbert, Gordon, Subrahmanyam, and Green (3) purified aldolase from rabbit muscle and obtained an amorphous product which was electrophoretically homogeneous (4). Warburg and Christian (5) crystallized aldolase from rat muscle and reported a value for the "turnover number" which was about twice that found by Herbert *et al* for their best preparation.

Seemingly unrelated to these observations was the isolation from rabbit muscle by Baranowski (6) of two crystalline proteins designated myogen A and myogen B. Baranowski assigned no enzymatic activity to these myogens and the methods he used appeared to differ from those used for the isolation of aldolase. He crystallized myogen A, in the form of hexagonal bipyramids, from ammonium sulfate solution at pH 6 and he stated that acetone could not be used successfully in the isolation of the protein. Warburg and Christian did use acetone in their fractionation procedure and they crystallized aldolase, in the form of thin six-sided plates, from ammoniacal ammonium sulfate solution. Herbert *et al* fractionated the enzyme with ammonium sulfate and stated that acetone inactivates even at 0°.

Engelhardt mentioned in a review (7) that aldolase activity had been observed in myogen A crystals. He suggested that myogen A may be identical with aldolase, but the turnover number he reported is quite low. Meyerhof and Beck (8) found that myogen A, prepared by Baranowski's procedure, showed aldolase activity that increased from about 0.1 unit per mg. of protein for the first crystals to about 0.7 unit per mg. of protein after the sixth recrystallization, but even this preparation had but one-sixth the activity reported by Warburg and Christian for crystalline rat aldolase. They concluded that myogen A is not aldolase.

\* This work was supported in part by a grant from the Rockefeller Foundation. A preliminary report was presented before the annual meeting of the American Society of Biological Chemists at Atlantic City, 1946 (1).

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In the course of fractionation of aqueous extracts of rabbit skeletal muscle, the fraction separating between 0.50 and 0.52 saturation with ammonium sulfate at pH 7.5 was observed to consist of fine crystalline needles (Fig 1). These could be recrystallized generally as needles, but upon occasion as large, well formed, elongated hexagonal plates (Fig 2). Hexagonal bipyramids which resembled myogen A (Fig 3) were obtained from rabbit muscle by a procedure which included fractionation with acetone and



FIG 1

FIG 1 Crystalline rabbit aldolase, needles dark-field, 600  $\times$

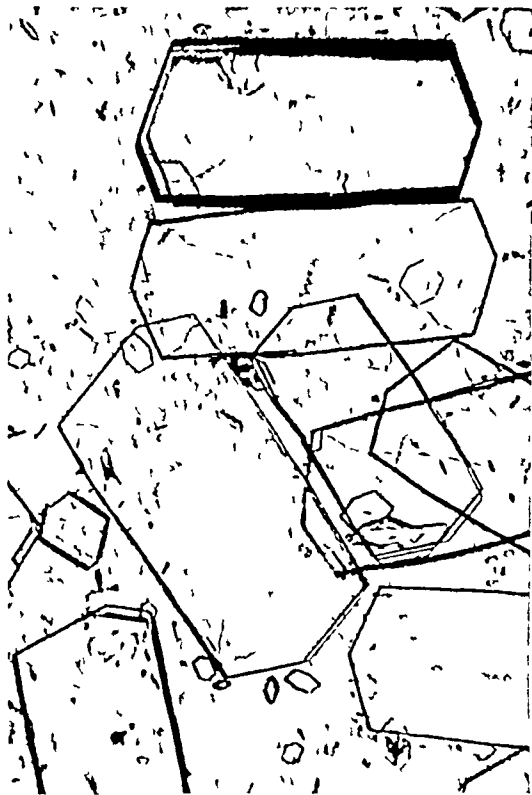


FIG 2

FIG 2 Crystalline rabbit aldolase, plates, 150  $\times$

differed in other respects from the method outlined by Baranowski (6), except in the method of crystallization from ammonium sulfate solution at pH 5.8. Each of these three crystalline proteins proved to possess high aldolase activity.

The bipyramids could be converted into needles and the needles, or the hexagonal plates, into bipyramids by crystallization from ammonium sulfate at pH 7.5 and 5.8 respectively. The aldolase activity of each of the three crystalline forms was the same and did not change upon inter-conversion.

Crystalline aldolase was also prepared from rat muscle (Fig 4) by the method of Warburg and Christian (5). The aldolase activity of this preparation was the same as that of the rabbit protein when either of two test systems was used, one based on chemical analysis, the other on optical measurements.

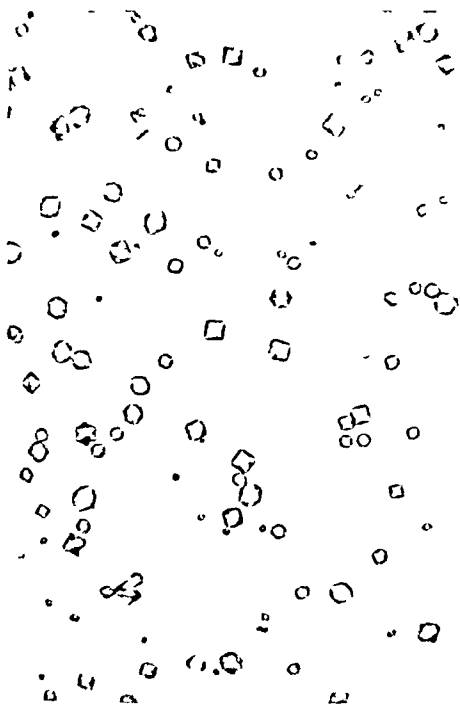


FIG 3

FIG 3 Crystalline rabbit aldolase, hexagonal bipyramids prepared from needles, 150  $\times$



FIG 4

FIG 4 Crystalline rat aldolase, 150  $\times$

#### EXPERIMENTAL

*Aldolase from Rabbit Skeletal Muscle, Crystallization As Needles*—This method, because of its simplicity and reproducibility, is recommended for the isolation of aldolase. Over 60 preparations according to this method have been carried out in this laboratory. The ground skeletal muscle of a rabbit is extracted in a cold room at 5° with two equal portions of cold water (or cold 0.03 N NaOH or KOH) and strained through gauze. The cold extract is first brought to pH 7.5 with dilute NaOH and then to 0.5 saturation by the addition of an equal volume of  $(\text{NH}_4)_2\text{SO}_4$  solution (saturated at room temperature and adjusted to pH 7.5 by the addition

of concentrated  $\text{NH}_3$ ) The solution is cooled to  $0^\circ$  and the precipitate is removed by filtration in the cold room To the clear filtrate is added enough saturated  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 7.5, to make the saturation 0.52 (4 ml of  $(\text{NH}_4)_2\text{SO}_4$  solution for each 100 ml of filtrate) The solution is stirred frequently while standing in the cold After some hours, often 12 or more, the presence of fine crystals can be detected by the "sheen" when the solution is stirred, the crystals do not settle on standing Initial crystallization may be speeded by allowing the solution to warm to room temperature, followed by a return to the cold room Further standing for several days is advisable for a maximum yield The crystals

TABLE I  
*Crystallization of Aldolase, Sample Protocol*

		Protein	Enzyme activity	Units per mg protein
		gm	units	
Extract from 500 gm of rabbit skeletal muscle		24.0	2136	0.089
Fractionation with $(\text{NH}_4)_2\text{SO}_4$	Saturation			
	0 - 0.40	4.1	131	0.031
	0.40 - 0.50	0.6	91	0.152
	0.50 - 0.52*	2.43	1631	0.671
	Supernatant fluid	17.1	205	0.012
Total recovered		24.2	2058	
0.50 - 0.52 fraction, recrystallized				0.684

\* Crystalline

can then be removed by filtration or high speed centrifugation The sample protocol in Table I shows that the separation is sharp, and that the activity of the first crystals is high and increases but slightly on recrystallization The yield of first crystals corresponds to about 75 per cent of the aldolase activity in the crude aqueous extract and amounts to 10 per cent of the extracted proteins or 0.35 gm per 100 gm of muscle

*Recrystallization*—The precipitate of fine needles is dissolved in a little water and brought to pH 7.5 A small amount of amorphous material may be removed by bringing the solution to incipient turbidity with  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5 and letting it stand for several hours in the cold Crystals begin to separate upon the cautious addition of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 7.5, at less than 0.5 saturation in the cold The protein can be recrystallized at room temperature in the same way Since this

protein is less soluble at room temperature than in the cold, at concentrations of  $(\text{NH}_4)_2\text{SO}_4$  higher than about 0.4 saturation, it can also be recrystallized with the aid of this property.

These fine crystals are difficult to see under the microscope, since the contrast is slight. It has been impossible to make certain that their appearance as needles is not misleading, and that they are not, in reality, extremely thin plates seen on edge. Their appearance in a dark field, of which Fig. 1 is an inadequate representation, seems to indicate needles.

*Crystallization As Plates*—When aldolase needles are dissolved in water, brought in the cold to incipient turbidity with  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5 (about 0.4 saturation), and allowed to warm gradually to room temperature, the protein has occasionally been found to separate as beautiful large six-sided plates (Fig. 2). The plate form has been recovered upon recrystallization, with seeding, but it has not proved possible to convert the needle form to the plates every time it has been attempted. The factors which control this behavior have not been determined. The plates as well as the needles can be converted to bipyramids (see below).

*Crystallization As Bipyramids*—These crystals were obtained from rabbit muscle as a by-product in the purification of the enzyme phosphoglucumutase. An extract was prepared as described above and all steps of the fractionation were carried out in a cold room at 5°. The precipitate that formed between 0.4 and 0.6 saturation with  $(\text{NH}_4)_2\text{SO}_4$  at pH 7 was collected by filtration and dissolved in 1 per cent sodium glycerophosphate solution (5 volumes of solution to 1 volume of precipitate). To this solution, cooled to 0°, ice-cold acetone was slowly added to a concentration of 41 per cent, the precipitate was discarded by centrifugation, and the concentration of acetone in the supernatant fluid brought to 55 per cent. After the second acetone precipitate was dissolved in 1 per cent glycerophosphate solution, the successive fractionation with  $(\text{NH}_4)_2\text{SO}_4$  (between 0.4 and 0.6 saturation) and with acetone (between 40 and 55 per cent) was repeated. The acetone precipitate was dissolved in 0.3 saturated  $(\text{NH}_4)_2\text{SO}_4$ , pH 7, insoluble material was discarded, and the supernatant fluid was brought to 0.45 saturation. It was at this step in the procedure that aldolase could be separated from phosphoglucumutase. The cold 0.45 saturated  $(\text{NH}_4)_2\text{SO}_4$  solution of protein was warmed to 25°, with constant stirring, by being placed in a water bath at 30°. The heavy precipitate that formed (which could be redissolved completely by cooling the solution again to 0°) was separated by centrifugation at room temperature and dissolved in a small volume of ice-cold water. To the clear solution, saturated  $(\text{NH}_4)_2\text{SO}_4$  solution (pH about 5.7) was added dropwise with stirring until a faint turbidity had developed. It was then left at 5°. Bipyramidal six-sided crystals appeared after several days. Further addi-

tion of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution led to further crystallization. These crystals were very large and were free of visible amorphous "background" upon microscopic observation<sup>1</sup>. The crystals were centrifuged in the cold and brought into solution in ice-cold water in which they dissolve very slowly. Recrystallization from a solution containing 1 per cent protein, or more, was rapid when saturated  $(\text{NH}_4)_2\text{SO}_4$  solution was added to faint turbidity and the solution was seeded. The second crystals were smaller than the first.

The hexagonal bipyramids shown in Fig. 3 were actually prepared from aldolase needles, but cannot be distinguished by ordinary visual observation from the recrystallized bipyramids prepared as described above. The several varieties of hexagonal bipyramids, described by Chrobak and Baranowski (9) for the preparation of myogen A, have not been detected among our crystals.

*Interconversion*—To a clear solution, prepared from the aldolase needles and containing about 2 per cent protein, saturated  $(\text{NH}_4)_2\text{SO}_4$  solution that has not been neutralized (about pH 5.7) is added in the cold until a very faint turbidity persists. This is ordinarily sufficient to bring the entire solution to pH 6 or slightly less. Typical hexagonal bipyramids separate in 20 to 48 hours if the solution is seeded (Fig. 3). These crystals, or the bipyramids prepared directly from the muscle, dissolved in water, can easily be converted to needles by crystallization from  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5.

*Crystalline Aldolase from Rat Skeletal Muscle*—The skeletal muscle from nine rats, amounting to 780 gm., was ground and extracted with water. All subsequent operations were performed at 0–4° and followed the procedure outlined by Warburg and Christian (5), except as mentioned. After the preliminary fractionation with acetone had been completed, it was found that the protein was more soluble in the indicated concentrations of  $(\text{NH}_4)_2\text{SO}_4$  at 0–4° than was anticipated from Warburg and Christian's directions, while upon raising the temperature of the solution to about 20° much of the protein precipitated. This fraction was separated by centrifugation at room temperature and preserved, since the precipitation on warming resembled the behavior of rabbit aldolase. Fractionation of the rest of the material according to Warburg and Christian (5) was continued at low temperature. After two fractionations with ammoniacal  $(\text{NH}_4)_2\text{SO}_4$ , instead of the four which they described, it proved possible to crystallize both the fraction separated by warming and the one fractionated at low temperature. The crystallization was accomplished with ease by adding saturated ammoniacal  $(\text{NH}_4)_2\text{SO}_4$  solution to the ice-cold concen-

<sup>1</sup> In our experience the first myogen A crystals obtained by the Baranowski procedure (6) are usually contaminated with a considerable amount of amorphous material, which continues to appear on successive recrystallizations.

trated protein solution until faint turbidity developed and then allowing the solution to warm slowly to room temperature. Recrystallization followed the same procedure. The crystals (Fig. 4) have the same characteristic appearance as those shown in the paper by Warburg and Christian (5).

We did not investigate in this preparation whether any of the other steps in the Warburg and Christian procedure might be eliminated, except that the protein separated by warming crystallized without having been treated with alumina cream, these crystals had the same aldolase activity as those prepared by the more elaborate method, including alumina cream treatment.

*Aldolase Activity, Chemical Test*—The chemical test used by Herbert *et al.* (3) has been modified for convenience in several particulars. The substrate solution contains 0.01 M fructose-1,6-diphosphate<sup>2</sup> and 0.1 M glycine buffer at pH 9.0. The enzyme solution is prepared by dilution with 0.1 M KCN solution acidified to pH 9.0<sup>3</sup>. To 0.1 ml of substrate solution in a Pyrex test-tube in a bath at 30° is added 0.1 ml of enzyme solution and the reaction is allowed to proceed for 5 minutes. It is stopped by the addition of 1.0 ml of 1 N NaOH, freshly prepared each day (to avoid SiO<sub>2</sub>). After 15 minutes at room temperature, for the hydrolysis of the alkali-labile triose phosphate formed in the reaction, the solution is neutralized with an equivalent amount of H<sub>2</sub>SO<sub>4</sub>. The reagents for the determination of inorganic phosphate according to Fiske and Subbarow (11) are then added directly, the final volume being 10 ml. The color is measured in the Klett photocolormeter with Filter 660.

*Optical Test*—The crystalline D-glyceraldehyde phosphate dehydrogenase from rabbit muscle (12) has been used, instead of the corresponding yeast enzyme, in the optical test described by Warburg and Christian (5). Since the dehydrogenase is present in excess, the aldolase concentration determines the rate of the reaction. The rate of reduction of diphosphopyridine nucleotide (DPN) is followed by measuring the absorption of light at 340 mμ with the Beckman spectrophotometer. The concentrations of reagents are those used by Warburg and Christian, except that of DPN, which is lower. Tests showed that when DPN was added in one-half the usual concentration this did not affect the rate. The concentration of DPN has been determined at 340 mμ after complete enzymatic reduction by glycerol-

- Fructose-1,6 diphosphate (a commercial sample of the calcium salt obtained from the Schwarz Laboratories) gave a high blank in the chemical test. It was purified by precipitation as the monobarium salt (10) and converted to a solution of the sodium salt for use.

<sup>3</sup> In order to extend the range of proportionality of enzyme action on dilution, it is desirable to add a protective protein. 0.2 mg of recrystallized human serum albumin in 1 ml of cyanide solution has been found satisfactory.

dehyde phosphate in the presence of the dehydrogenase and arsenate Ohlmeier's value of the extinction coefficient (13) was used in the calculations

The value of the aldolase activity at a fixed temperature is not easily obtained in the optical test, since the temperature rises considerably within the absorption cell of the Beckman spectrophotometer as the latter is constructed at present. The temperature has been measured within the cell at the end of each experiment and the data have been corrected to 30° with the aid of the temperature coefficients given by Herbert *et al* (3). Concordant values upon duplicate experiments are thus obtained.

Protein concentrations in solutions of crystalline aldolase were determined optically, from the absorption of light at 280 m $\mu$  measured in the Beckman spectrophotometer. Protein concentration (mg per ml) =  $(-\log_{10} T)/Kl$  where  $K = 0.806$  and  $l$  is in cm. Protein concentrations, especially in crude fractions, were also determined by the quantitative biuret method of Robinson and Hogden (14).

The results have been expressed in Table II both in aldolase units and as a turnover number. 1 unit represents 1 mg. of P transformed in 1 minute, under the experimental conditions shown in Table II. The turnover number has been calculated in terms of the number of moles of hexose diphosphate transformed in 1 minute by 150,000 gm. of protein.<sup>4</sup> Since each of the previous workers had used somewhat different methods of expressing activity, the results from the literature given in Table II have been recalculated in the terms just described.

No details are given by Engelhardt (7) as to how the value for the turnover number of aldolase was obtained, but in any case the value is very low. The turnover number given for myogen A by Meyerhof and Beck (8) at 37° is the same as that we have found at 30°. Since the temperature coefficient,  $Q_{10}$ , is close to 2 (3), it follows that the protein described in this paper is considerably more active than repeatedly recrystallized myogen A.

According to their chemical test, the preparations of Herbert *et al* (3) appear to be more active at 30° than our preparations, but this is probably due to differences in the methods used for measuring activity rather than in the purity of the preparations. That the chemical test may need reexamination is indicated by the fact that we have obtained at pH 9 in the chemical test the same activity as at pH 7.6 in the optical test. Since aldolase is about 25 per cent more active at pH 9 than at pH 7.6 (3), it would appear that our chemical test gives values which are too low.

<sup>4</sup> This figure was chosen to represent the molecular weight on the basis of Grålen's results with myogen A (15). The minimum molecular weight calculated from amino acid analyses of aldolase (16) is of the same order of magnitude and the diffusion constants of the two proteins agree closely.

Warburg and Christian (5) have pointed out that their rat aldolase preparations were twice as active as the rabbit aldolase preparations of Herbert *et al* (3). This might have been due to species difference, but the data in Table II show that our rat and rabbit preparations have the same activity, both in the chemical and in the optical tests.

With the same optical method as that used by Warburg and Christian we found at 30° about 40 per cent of the activity, for crystalline rat aldolase, that might have been expected from the measurements of these authors at 20° and 38°. We have explored the effect of variations in concentrations of substrate and other components of the reaction mixture without detect-

TABLE II  
*Aldolase Activities of Various Preparations*

Investigators	Source	<i>t</i>	pH	Chemical test	Optical test	Turnover No
		°C		units per mg protein	units per mg protein	
Engelhardt (7)						1,000*
Meyerhof and Beck (8)	Rabbit	37	†	0.7		1,690
Herbert <i>et al</i> (3)	"	30	7.3	1.1†		2,680†
		38	7.3	2.0		4,950
Warburg and Christian (5)	Rat	20	7.6		0.89	2,150
		38	7.6		4.31	10,430
This investigation	Rabbit	30	7.6		0.70	1,690
		30	9.0	0.69		1,670
	Rat	30	7.6		0.73	1,770
		30	9.0	0.69		1,670

\* Not recalculated. Details of the original measurement and calculation not given (7).

† pH not clearly stated but presumably near 7.6.

‡ Estimated from Fig. 3, Herbert *et al* (3).

ing significant differences. In particular, the presence of cysteine is necessary for the full activity of the glyceraldehyde phosphate dehydrogenase from muscle (12), which we used in the optical test. Warburg and Christian (5), who used the corresponding enzyme from yeast, state that in their optical test cysteine was ordinarily used, except when the aldolase had been crystallized. To avoid the possibility that the known reaction of cysteine with glyceraldehyde phosphate might have interfered, we have substituted glutathione with identical results.

The reason for the discrepancy between the values of Warburg and Christian and our own has not been explained. The data given in the next section show that the proteins from both rat and rabbit muscle proved to be electrophoretically homogeneous over a wide pH range.

In view of the fact that the same activity was obtained for each of the



three crystalline modifications of the rabbit protein, that the activity remained the same on interconversion of these different crystal forms, and that the same activity was also obtained for rat aldolase crystallized according to Warburg and Christian, we are of the opinion that these proteins represent the enzyme aldolase. Further work is in progress to examine the exact relation between the myogen A preparations of Baranowski and our preparation of aldolase.

*Electrophoresis*—The electrophoretic mobility and homogeneity of crystalline preparations of aldolase have been investigated in the Tschus apparatus (17) equipped with the long center section (Longworth *et al* (18)). The cylindrical lens optical system (Philpot (19)) has ordinarily been used. Experiments have been performed at 2°, over the pH range 5.2 to 8.6, in acetate, phosphate, or veronal buffers of ionic strength,  $\Gamma/2$ , 0.1. Phosphate buffers were prepared according to Green (20), all were checked with a glass electrode pH meter, sensitive to  $\pm 0.02$  pH. The pH of 0.05 M potassium acid phthalate was taken as 4.00 (at about 20°).

From a comparison of the electrophoretic schlieren diagrams for the rabbit muscle extract and for aldolase (Fig. 5, *a* and *b*), it may be noted that aldolase forms part of the large protein component that moves most slowly at pH 7.4. D-Glyceraldehyde-3-phosphate dehydrogenase also forms part of this component (12).

Fig. 5, *b*, obtained with first crystals from rabbit, shows a main component and a small, fast moving peak, which is absent after recrystallization, while the main peak remains unchanged. On the descending schlieren diagram of Fig. 5, *b*, there will be noted a thin "spike" on the side of the main peak. This has appeared in every electrophoretic diagram of recrystallized rabbit or rat aldolase on the descending side only.

The appearance of the spike is similar to that of the so called " $\beta$ -anomaly" observed in electrophoretic diagrams obtained with blood plasma (21). In a similar fashion the spike appears to be associated with the appearance of a thin plane of turbidity which can just be detected at the descending boundary by direct inspection of the cell. The appearance of the spike during electrophoresis may mean that aldolase itself precipitates under the influence of the conditions that obtain in the descending boundary. There is no evidence at present to implicate a lipid or lipoprotein, extraction of aldolase with ether in the cold is without effect on the spike, while the analytical data on the dried protein leave little room for such a component (16).

With the exception of the spike phenomenon, the recrystallized rabbit protein showed a high degree of electrophoretic homogeneity over the pH range 5.2 to 8.6.

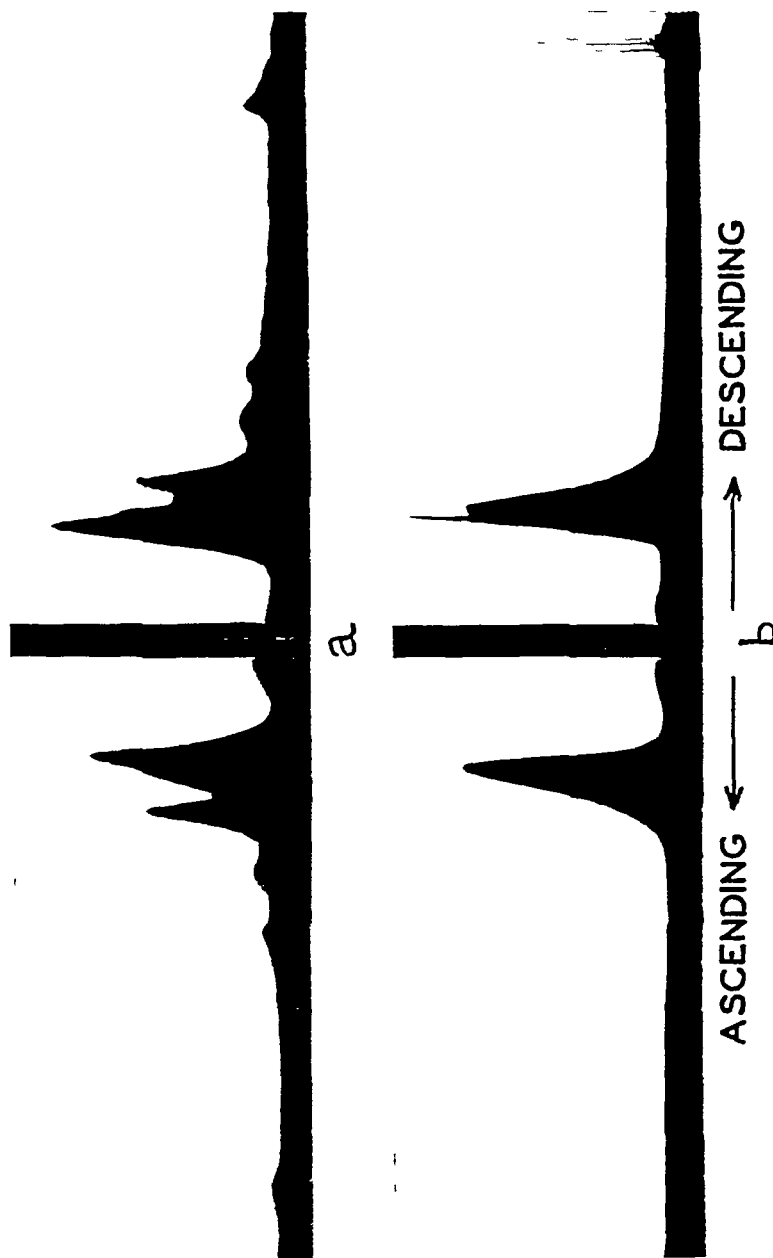


FIG 5 Electrophoretic sedimentation diagrams in phosphate buffer, pH 7.4, ionic strength 0.1, after 1 hour at 2°  
*a*, original extract of muscle, *b*, fraction crystallized between 0.50 and 0.52 saturation with  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5

Fig 6 represents the variation in the electrophoretic mobility of rabbit aldolase from pH 5.7 to 7.8. The ascending and descending mobilities (corrected for cylindrical lens error) are generally quite close, and have been averaged. In several instances rabbit aldolase crystallized both as needles and as bipyramids proved to have the same mobility under identical conditions. The points have been connected by a smooth free-hand curve, establishing the isoelectric point under these conditions at pH 6.05. Bate-Smith (4) determined the electrophoretic mobility of a purified aldolase prepared by Herbert *et al* (3) at pH 6.0 and 7.0 and estimated the isoelectric point to be about pH 6.3.

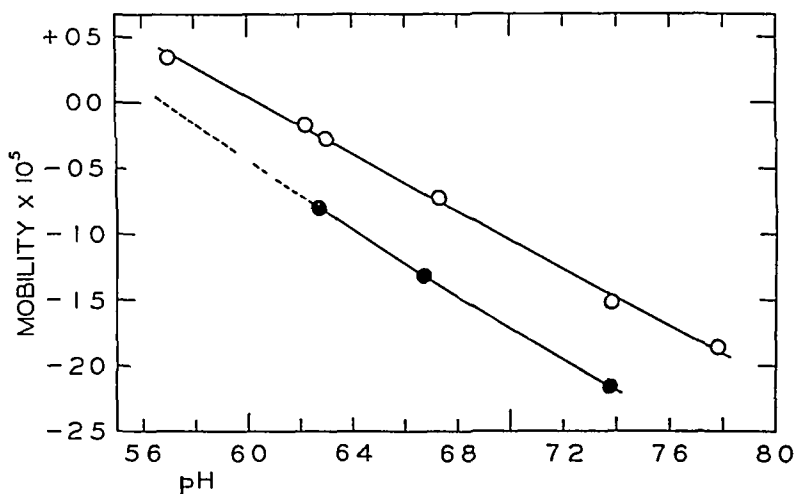


Fig 6 Electrophoretic mobility of crystalline aldolase in sq cm volt<sup>-1</sup> sec<sup>-1</sup>  $\times 10^5$  at 2° in phosphate buffers of ionic strength 0.1, ○ rabbit, ● rat

The mobility of rat aldolase is considerably greater than that of rabbit aldolase at three pH values and under the same conditions. Extrapolation, as indicated in Fig 6, would give the isoelectric point of the rat protein as pH 5.7. The rat aldolase is also electrophoretically homogeneous over this pH range.

Mobilities observed in acetate buffer, pH 5.2 and 5.6, and in diethyl barbiturate buffer, pH 8.6, do not fall on the curve for phosphate buffer shown in Fig 6. Specific effects of buffer species on mobility have been reported in other instances (*e g*, Davis and Cohn (22)).

**Diffusion**—The diffusion constant of rabbit aldolase has been measured in the electrophoresis cell with long center section as described by Longworth (23) and tested in this laboratory by Green (24). Phosphate buffer,  $\Gamma/2 = 0.1$ , pH = 7.1, was used and the experiments were performed at 2°. Two separate experiments were carried out. The results were computed separately for each of the two limbs of the cell, from enlarged

tracings of schlieren scanning photographs, according to the relations  $D_t = .1^2/4-tH_m^2$  and  $D_\mu = \mu^2/2t$ , where  $A$  is the area under the diffusion diagram in sq cm,  $t$  is the time in seconds,  $H_m$  is the maximum height of the curve in cm, and  $\mu$  is one-half the breadth of the curve, in cm, at its inflection point, where  $H_\mu = H_m/\sqrt{e}$ . The values obtained at a number of different times were essentially constant during an experiment lasting 220 hours. The method of moments, applied to a few individual diagrams, gave values consistent with those obtained by the other two methods. A few curves were also compared with normal distribution curves, with satisfactory agreement (25).

The average value in water at 20° was for  $D_t$  4.58 and for  $D_\mu$  4.68  $\times 10^{-7}$  sq cm sec<sup>-1</sup>. The average, 4.63, is quite close to the value of  $D_{20w} = 4.78 \times 10^{-7}$  sq cm sec<sup>-1</sup> obtained by Gial  n in a study of the physical constants of crystalline myogen A from rabbit muscle (15).

The authors wish to thank Mr. Robert Loeffel for assistance in carrying out a number of the diffusion and electrophoresis measurements reported in this paper.

#### SUMMARY

1 The enzyme aldolase has been isolated from rabbit skeletal muscle in the form of fine needles or hexagonal plates by crystallization from  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5 or in the form of hexagonal bipyramids by crystallization from  $(\text{NH}_4)_2\text{SO}_4$  at pH 5.8. These crystal forms can be interconverted by crystallization at the appropriate pH without change in the specific aldolase activity. The turnover number corresponds to the splitting of 1670 moles of fructose diphosphate by 150,000 gm of protein in 1 minute at 30° and pH 7.6. Aldolase crystallized from rat muscle has the same turnover number.

2 About 75 per cent of the aldolase activity and about 10 per cent of the protein in an aqueous extract of rabbit muscle can be recovered as aldolase crystals by fractionation between 0.50 and 0.52 saturation with  $(\text{NH}_4)_2\text{SO}_4$  at pH 7.5.

3 Both rabbit and rat aldolase are electrophoretically homogeneous over a wide pH range. The isoelectric points in phosphate buffer of ionic strength 0.1 are 6.05 and 5.7, respectively.

4 The diffusion constant of crystalline rabbit aldolase is 4.63  $\times 10^{-7}$  sq cm sec<sup>-1</sup> in water at 20°.

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# CRYSTALLINE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE\*

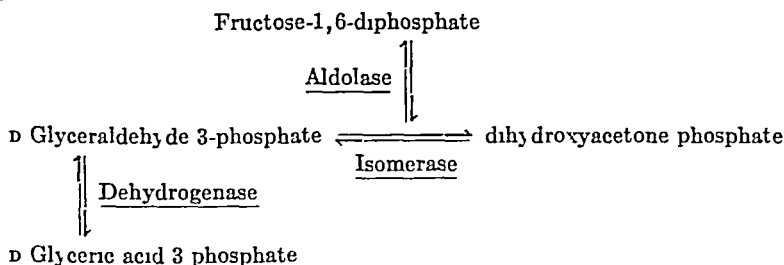
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The reaction catalyzed by this enzyme consists in the oxidation of a —CHO to a —COO PO<sub>3</sub>H<sub>2</sub> group and represents the first oxidative step in the degradation of carbohydrate in the tissues. Diphosphopyridine nucleotide (DPN) and inorganic phosphate are necessary for this reversible enzymatic reaction. The equilibrium has been investigated by Warburg and Christian (1), Drabkin and Meyerhof (2), and Meyerhof and Oesper (3). When inorganic phosphate is replaced by arsenate, the reaction becomes irreversible. In both instances the reaction can be followed by measuring the appearance of reduced DPN spectrophotometrically at 340 mμ, a method originally introduced by Warburg and Christian.

The substrate for this enzyme is formed from fructose-1,6-diphosphate through the action of aldolase, the enzyme described in the preceding paper



This sequence of reactions, originally proposed by Embden and Meyerhof, could be demonstrated by means of the crystalline enzyme preparations because they are free of isomerase (Fig 1). In the presence of aldolase, dehydrogenase, arsenate, and DPN, glyceraldehyde phosphate only disappears and the yield is 1 mole of triose phosphate oxidized per mole of hexose diphosphate added, when, in addition, a purified preparation of triose phosphate isomerase (5) is added, dihydroxyacetone phosphate disappears and a second mole of triose phosphate is oxidized.<sup>1</sup>

\* This work was supported in part by a grant from the Nutrition Foundation, Inc.

<sup>1</sup> This provides a sensitive method for the quantitative determination of hexose diphosphate and triose phosphate, either separately or combined, depending on the order of addition of the three enzymes. This method will be described in a subsequent publication.

The preparation of the crystalline enzyme was described in a preliminary report (6) and shortly thereafter a note appeared by Dixon and Caputto (7) in which crystallization was achieved by another method of preparation. The enzyme had previously been crystallized from yeast by Warburg

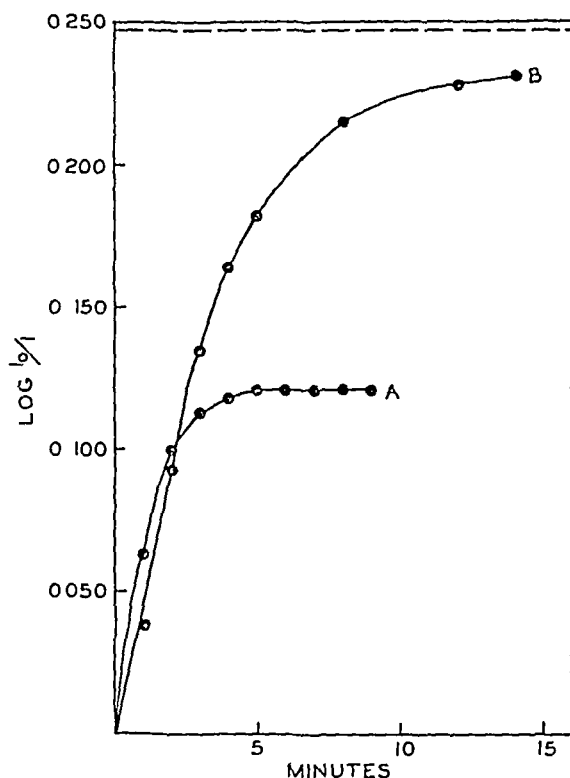


FIG. 1 Spectrophotometric measurement of triose phosphate oxidation with fructose diphosphate as substrate. Curve A, the reaction mixture consisted of fructose diphosphate, aldolase, dehydrogenase, DPN, and arsenate. 1 mole of triose phosphate was oxidized (or DPN reduced) per mole of fructose diphosphate added. Curve B, the reaction mixture contained in addition triose phosphate isomerase. 2 moles of triose phosphate were oxidized per mole of fructose diphosphate. The dotted line indicates the amount of reduction of DPN expected from the amount of fructose diphosphate added. Fructose diphosphate was determined by the phenylhydrazine method of Deuticke and Hollmann (4).

and Christian (1). In confirmation of them it was found that the enzyme, when used in much higher concentration, also oxidizes D-glyceraldehyde.

The present paper contains details of preparation, data on the properties of the enzyme protein, and kinetic measurements, while the papers which follow contain data on the prosthetic group and on amino acid composition. The enzyme constitutes about 7 to 12 per cent of the extracted proteins and the yield is about 300 mg per 100 gm of muscle.

## EXPERIMENTAL

*Method of Preparation*—A rabbit is injected intravenously with a lethal dose of amytal. It is rapidly skinned and the leg and back muscles are excised and weighed. All further steps are carried out in a cold room. The muscles are passed through a meat grinder, extracted immediately with 1 volume of 0.03 N KOH for 10 minutes with occasional stirring, and strained through gauze. The extraction is repeated and the residue is suspended in 0.5 volume of water for 5 minutes and strained as above. The pH, measured with the glass electrode in the combined extracts, varied in six different preparations from 6.6 to 7.2.

If it is desired to prepare both aldolase and dehydrogenase, 1 volume of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 7.5 to 7.8 (saturated at room temperature and pH adjusted with ammonia), is added to the extract (0.5 saturation) and the mixture placed in an ice bath for about 0.5 hour. From the time of killing the rabbit to the addition of the salt solution not more than 1.5 hours should elapse. The mixture is filtered through folded paper (Whatman No. 1), and to the clear filtrate more saturated  $(\text{NH}_4)_2\text{SO}_4$  solution is added to bring the saturation to 0.52 (4 ml. to each 100 ml.). The pH should be 7.6 to 7.8. This solution either is left at 5° for 2 to 3 days or is warmed up very slowly to 20°, then returned to 5°. This procedure speeds up the rate of crystallization of aldolase (8). When a heavy mass of aldolase crystals (which do not settle) has formed, they are separated by filtration or centrifugation.

When it is not desired to prepare aldolase, saturated  $(\text{NH}_4)_2\text{SO}_4$  solution is added to 0.52 saturation in one step, followed immediately by filtration or by centrifugation in a high speed angle centrifuge.

To each 100 ml. of filtrate at 0.52 saturation are added 13 gm. of solid  $(\text{NH}_4)_2\text{SO}_4$ , which brings the saturation to 0.72. As soon as the salt is dissolved by gentle stirring, the solution is filtered through folded paper, the filtrate being poured back on the filter until it is perfectly clear. Filtration is fairly rapid and the pH of the filtrate is about 7.5. To the filtrate, 15 per cent ammonium hydroxide solution is added dropwise with shaking until the pH is 8.2 to 8.4. The pH is measured with a glass electrode, or with metacresol purple as indicator, in an aliquot of the 5 times diluted filtrate. Crystals appear in several hours, and even without seeding a large crop of crystals (which do not settle) forms overnight. The suspension of the crystals may be left standing for several days to increase the yield. The crystals are separated by filtration through folded paper (Whatman No. 1). Filtration is rather rapid at first but eventually slows down, by the use of an automatic filtration device all the material from 300 to 500 gm. of muscle will pass through a filter of 24 cm. diameter overnight. The still moist crystalline precipitate is scraped off the paper with a spatula.



and dissolved in 40 to 80 ml of water. Paper fibers,  $\text{MgNH}_4\text{PO}_4$  crystals, and shreds are removed by centrifugation. To the clear, slightly reddish yellow solution are added 2 volumes of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 8.2 to 8.4, for each volume of water used in the solution of the crystals. There is no immediate precipitation of protein. In less than 1 hour, crystals begin to appear and in 24 hours a thick shimmering suspension of crystals has formed. Further recrystallizations are carried out in the same manner. The yellowish color is eventually removed with the mother liquors and the suspension appears pure white. By this procedure thirty-four preparations of the enzyme have been made in the past 2 years. There

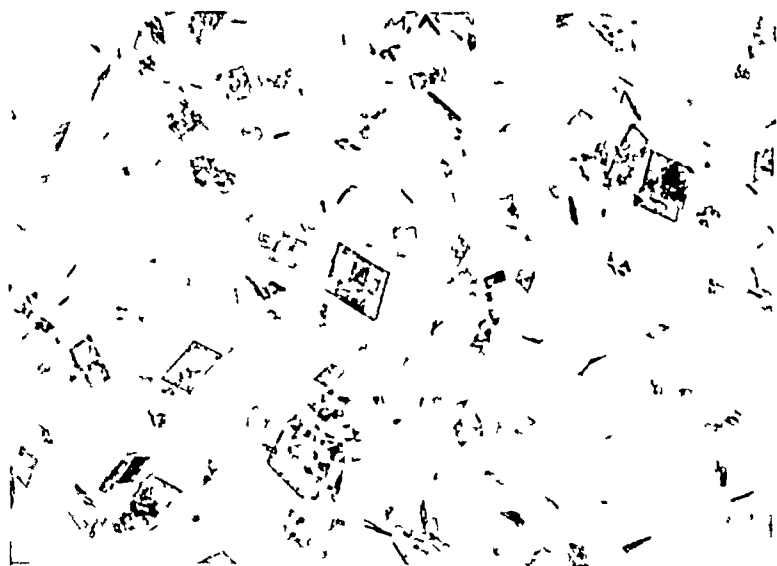


FIG. 2 Crystalline D glyceraldehyde-3-phosphate dehydrogenase,  $\times 260$

was no case of failure and the yield was 1 gm. or more of enzyme per rabbit.<sup>2</sup>

The first crystals usually appear in the form of rosettes, which are apparently made up of clusters of fine needles. Closer observation reveals that the crystals consist of diamond-shaped plates which stand on edge. On recrystallization the crystals remain separate. Because of the thinness of the plates and the closeness of the refractive indices of the crystals and the mother liquor, satisfactory photomicrographs were obtained only when the crystals were stained with methylene blue. The crystals shown in Fig

<sup>2</sup> It should be emphasized that, in order to obtain a good yield of crystalline dehydrogenase, the rapidly excised muscles must be ground and extracted without delay. When the muscles were left at 5° for 1 hour, or when 0.5 hour elapsed between the time the muscles were ground and placed in the extraction fluid, no, or only a small, yield of crystalline enzyme could be obtained.

2 were obtained by allowing the enzyme to recrystallize slowly from 0.6 saturated  $(\text{NH}_4)_2\text{SO}_4$  solution and are larger than those ordinarily obtained from 0.66 saturated  $(\text{NH}_4)_2\text{SO}_4$ . Both the diamond shape of the crystals and the simulated rod shape due to the crystals standing on edge are seen in the photograph.

An example of the yield and activity on recrystallization is given in Table I. It may be seen that the enzyme crystallizes from a 0.14 per cent protein solution in which it constitutes about one-half of the protein present. About 10 per cent of the enzyme is lost in the mother liquor, while on recrystallization from more concentrated solutions the loss is 5 to 8 per cent. The specific activity of the crystals increases slightly on recrystallization, while that of the mother liquor increases very markedly and approaches

TABLE I  
*Recrystallization of Dehydrogenase*

The enzyme was prepared from 500 gm. of rabbit muscle by the method described in the text.

Crystallization	Protein in crystals	Mother liquor		Specific activity	
		Volume	Protein content	Crystals	Mother liquor
	gm	ml	gm		
1st	1.56	2100	1.42	$9.15 \times 10^8$	$0.94 \times 10^8$
2nd	1.43	206	0.13	$8.64 \times 10^8$	$2.92 \times 10^8$
3rd	1.35	214	0.08	$10.1 \times 10^8$	$6.66 \times 10^8$

\* The specific activity (at 25°) was obtained by dividing the bimolecular rate constants by the mg. of protein per ml. of reaction mixture.

that of the crystals. The enzyme is not completely stable when in solution in ammonium sulfate and this may account for the fact that the specific activity in the mother liquor did not reach that of the crystals.

Why 0.03 N alkali rather than water is used for the extraction of muscle in the preparation of the dehydrogenase is shown in the following experiment.

1 part of ground rabbit muscle was extracted with water (pH of extract 6.1) and another part with 0.03 N KOH (pH of extract 7.1), followed in each case by the procedure outlined for the preparation of the dehydrogenase. The water extract yielded only a trace of crystals, while a large crop was obtained from the alkaline extract. Both preparations were analyzed for protein and specific activity by sampling the well mixed 0.72 saturated  $(\text{NH}_4)_2\text{SO}_4$  solution. The water extract yielded 0.69 mg. of protein per ml. and a specific activity of  $5.12 \times 10^8$ , the respective values for the alkaline extract were 1.43 mg. of protein and  $8.55 \times 10^8$  specific

activity From this it may be calculated that the extract prepared with alkali contained 3.5 times more enzyme than the water extract, and it is therefore not surprising that few crystals were obtained from the latter Further investigation is required to explain the low yield of the enzyme from water extracts

*Electrophoresis*—The electrophoretic behavior of D-glyceraldehyde phosphate dehydrogenase has been investigated with the technique described for aldolase (8) Experiments have been performed at 2° over the pH range 5.1 to 8.45

The recrystallized enzyme is electrophoretically homogeneous over the pH range 6.2 to 7.7 Fig. 3, from an experiment at pH 7.4, shows a single peak exhibiting a slight skew It was found that the enzyme solution (7 mg per ml) had lost 7 per cent of its activity during the dialysis period

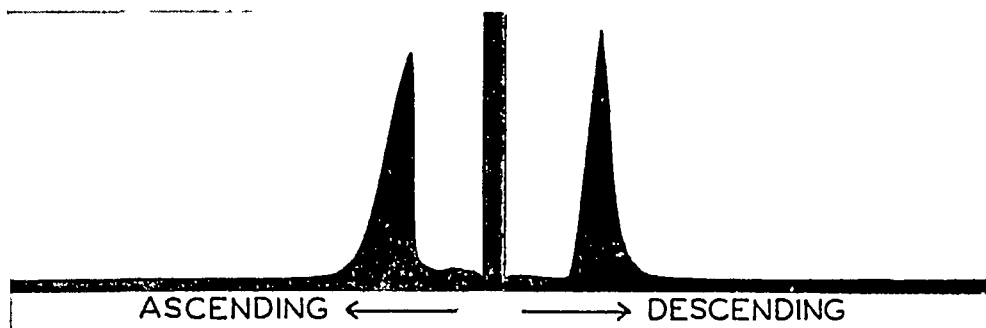


FIG. 3 Electrophoretic schlieren diagram of recrystallized D-glyceraldehyde 3-phosphate dehydrogenase after 4 hours at 2° in phosphate buffer, pH 7.4, ionic strength 0.1

of 20 hours which preceded the electrophoresis In solutions more acid than pH 6.2 or more alkaline than pH 7.7, instability of the enzyme interferes with electrophoretic measurements When the enzyme was dialyzed against acetate buffers of pH 5.1 to 5.4 for 20 hours, it lost about 70 per cent of its activity No appreciable turbidity developed, but a considerable part of the protein became insoluble in 0.3 saturated  $(\text{NH}_4)_2\text{SO}_4$  solution at pH 8.2, indicating denaturation During electrophoresis at this low pH range several minor peaks appeared besides a major one In veronal buffer of pH 8.45, pronounced turbidity developed during electrophoresis, nearly masking the moving boundaries

Fig. 4 shows the variation in electrophoretic mobility from pH 6.2 to 7.7 in phosphate buffer of ionic strength 0.1 The ascending and descending mobilities were generally quite close, and have been averaged A free-hand curve drawn through the points establishes the isoelectric point under these conditions at pH 6.55

*Activity Measurements*—A portion of the crystal suspension is centrifuged sharply, drained, and dissolved in 0.03 M sodium pyrophosphate, pH 8.5, in the cold to give a concentration not less than 1 mg of protein per ml. This stock solution is stable for 2 to 3 hours at 0°, but is unstable at room temperature. For activity tests this solution is diluted with pyrophosphate buffer containing cysteine. Dilution in the absence of cysteine results in loss of enzyme activity. Cysteine cannot be replaced by a protective protein such as crystalline serum albumin. To give an example (which incidentally describes the procedure usually followed), 0.1 ml of a stock solution (410  $\gamma$  of protein) was diluted to 4 ml: (a) with 0.03 M pyrophosphate, pH 8.5, (b) with 0.004 M cysteine in 0.03 M pyrophosphate, (c) with 0.03 M pyrophosphate containing 225  $\gamma$  of serum albumin, and

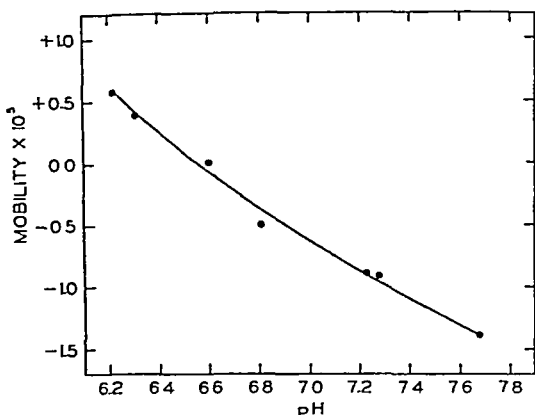


FIG. 4. Electrophoretic mobility of D-glyceraldehyde 3-phosphate dehydrogenase in sq. cm. volt<sup>-1</sup> sec<sup>-1</sup>  $\times 10^3$  at 2° in phosphate buffers of ionic strength 0.1

(d) with a solution containing cysteine, pyrophosphate, and albumin. As soon as each dilution was made, 0.1 ml (10  $\gamma$ ) was transferred to a silica absorption cell containing 2.7 ml of a 0.004 M cysteine-0.03 M pyrophosphate-DPN mixture. After the mixture stood for 7 minutes at room temperature, the reaction was started by the addition of 0.2 ml of a mixture of triose phosphate and arsenate. Density readings at 340  $m\mu$  were taken at 1 minute intervals in the Beckman spectrophotometer. Bimolecular rate constants were calculated from the 1 and 2 minute readings. These showed that Sample a had 45 and Sample c 23 per cent less activity than Samples b and d, which gave the same rate constant.

The usual composition of the reaction mixture in moles per ml was  $2.5 \times 10^{-7}$  for the D component of DL-glyceraldehyde phosphate,<sup>3</sup>  $1 \times 10^{-7}$

<sup>3</sup> We are indebted to Dr. H. O. L. Fischer, Dr. E. Baer, and Dr. H. A. Lardy for several samples of synthetic DL-glyceraldehyde phosphate (9). Determinations of

DPN,  $4.6 \times 10^{-6}$  arsenate,  $3 \times 10^{-5}$  pyrophosphate,  $4 \times 10^{-6}$  cysteine, pH 8.5. The reference cell contained the same reactants with the exception of the enzyme. The concentrations of triose phosphate and DPN are about 6 and 2.5 times higher, respectively, than those required to give one-half saturation of the enzyme. Bimolecular rate constants were calculated from the equation

$$K = \frac{2.3}{t(a-b)} \log \frac{b(a-x)}{a(b-x)}$$

where  $a$  is the initial concentration of triose phosphate,  $b$  that of DPN, and  $x$  the amount of reduced DPN formed in time  $t$  (minutes), all expressed in moles per ml. Reduced DPN was calculated from the spectrophotometric readings by means of the relation,  $(2.3 \log I_0/I)/1.45$ . This corresponds to the  $\beta$  coefficient for pure reduced DPN determined by Ohlmeyer (10). In some cases equal concentrations of triose phosphate and DPN were used in which case the equation reduces to

$$K = \frac{1}{t} \frac{x}{a(a-x)}$$

For protein determinations an aliquot of the stock solution was diluted with water and read immediately at the wave-length of maximum absorption (276 m $\mu$ ) in the spectrophotometer. The conversion factor, based on micro-Kjeldahl determinations, was 1.9, hence  $(2.3 \log I_0/I)/1.9 = \text{mg}$  of protein per ml. In some cases protein was determined by the biuret method of Robinson and Hogden (11) or, in the case of dialyzed enzyme solutions, according to the modification of this method by Weichselbaum (12). For comparison of specific activities, the rate constants were divided by mg of protein present per ml of reaction mixture.

Proportionality could be tested for only a limited range of enzyme concentrations (0.8 to 5  $\gamma$  per ml), because at higher enzyme concentrations the rate of reaction was too fast to permit accurate galvanometer readings. Within the range tested, proportionality was satisfactory. The bimolecular rate constant decreased somewhat with time. This was the case in the presence or absence of cysteine, as well as when glutathione was substituted for cysteine. For example, with 4  $\gamma$  of protein per ml the rate constants for 1, 2, and 3 minutes were 2.59, 2.49, and  $2.44 \times 10^6$ , respectively, giving a specific activity for the 1st minute of  $(2.59 \times 10^6)/0.004 = 6.5 \times 10^8$  at 24°.

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alkali-labile P or of the amount of DPN reduced enzymatically were used to calculate concentrations.

<sup>4</sup> In most of the experiments a sample of 50 per cent purity prepared in this laboratory was used.

An example of the activating effect of cysteine is given in Fig 5. Separate tests have shown that the effect of cysteine on the enzyme at room temperature is not instantaneous, maximum activity is reached in 5 to 7 minutes and is thereafter maintained for about 30 minutes, followed by a decline in activity after longer periods of incubation at room temperature. In order to test the enzyme under optimal conditions, it is diluted with and then kept for 7 minutes in the cysteine-pyrophosphate buffer in the presence

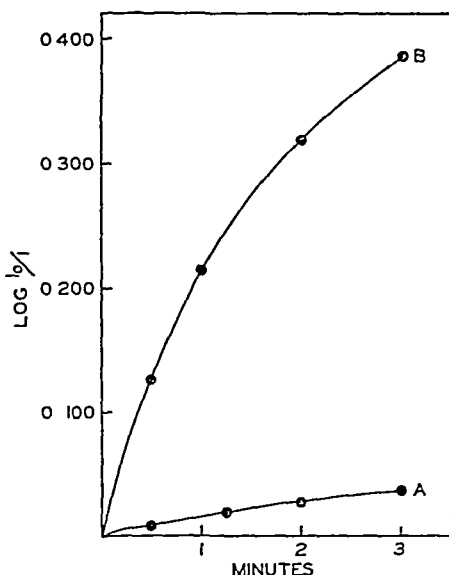


FIG 5 Activating effect of cysteine on glyceraldehyde phosphate dehydrogenase. In Curve A, a dilute solution of the enzyme ( $4 \gamma$  per ml) was kept for 10 minutes at  $25^\circ$  in the absence of cysteine. Curve A shows that the enzyme was practically inactive when tested in the absence of cysteine. Curve B shows that, when the same dilute solution of enzyme was pretreated with and tested in the presence of cysteine, it regained activity.

of DPN, the reaction is then started by the addition of triose phosphate plus arsenate.

Triose phosphate reacts with cysteine, but this source of error is small because of the low concentration of cysteine ( $0.004 \text{ M}$ ), because triose phosphate is added last, and because the reaction is measured for only 2 minutes. When  $2.5 \times 10^{-4} \text{ M}$  triose phosphate is incubated with  $4 \times 10^{-3} \text{ M}$  cysteine for 6 minutes at room temperature before the enzyme is added, the loss of triose phosphate reduces the rate of the reaction by about 20 per cent. Cysteine is a disturbing factor in the measurement of the equilibrium of the reaction in the presence of phosphate. After apparent

equilibrium has been reached, reduced DPN is slowly reoxidized, due to the removal of triose phosphate by cysteine. Glutathione reacts much more slowly with triose phosphate, as is shown by the fact that the equilibrium position is maintained unchanged for 10 minutes, furthermore the same equilibrium is reached in the presence and absence of glutathione. It has been found that glutathione is not as effective as cysteine in maintaining dilute enzyme solutions at the level of maximum activity, hence cysteine is preferred for rate, while glutathione is preferred for equilibrium measurements.

*Stability*—Enzyme crystals left suspended in 0.66 saturated  $(\text{NH}_4)_2\text{SO}_4$  and stored in the refrigerator lost 50 per cent of their original activity after 2.5 months. The small amount of enzyme which remains in solution in 0.66 saturated  $(\text{NH}_4)_2\text{SO}_4$ , pH 8.3, is stable for at least 24 hours at  $3^\circ$ .

In weak salt solution, stability of the enzyme is dependent, among other factors, on pH. An enzyme solution (1 mg per ml) was incubated for 30 minutes at  $30^\circ$  in acetate buffer at pH 5.5 and in pyrophosphate buffer at pH 7.3, the loss of enzyme activity was 27 per cent in the former and 17 per cent in the latter case. More dilute enzyme solutions are inactivated more rapidly under these conditions.

It has been found, in confirmation of Rapkine (13), that DPN exerts a protective effect on dilute enzyme solutions in the absence of cysteine. For example, an enzyme solution (2.5  $\gamma$  per ml) incubated at room temperature for 10 minutes in the presence of DPN, but in the absence of cysteine, retained 85 per cent of its activity when compared with a sample incubated with cysteine in the usual way. When incubated without DPN the enzyme was almost completely inactive.

*Dissociation Constants of Substrates*—The concentrations of triose phosphate and DPN were varied over a 10-fold range in enzymatic tests at pH 8.5. Satisfactory straight lines were obtained when the reciprocal of concentration was plotted against the reciprocal of the  $\log I_0/I$  reading at 1 minute. The values obtained from these graphs were  $5.1 \times 10^{-5}$  moles per liter for D-glyceraldehyde phosphate and  $3.9 \times 10^{-5}$  moles per liter for DPN. For the yeast enzyme, Warburg and Christian found  $3.2 \times 10^{-5}$  moles per liter for DPN at pH 7.5 with DL-glyceraldehyde as substrate. When recalculated by the method indicated above, the value would be about  $5 \times 10^{-5}$  moles per liter.

*Activity at Different pH Values and Turnover Number*—In these experiments, the enzyme was saturated with both glyceraldehyde phosphate and DPN<sup>5</sup> by using initial concentrations of each of  $4.8 \times 10^{-4}$  moles per liter. The initial rate of the reaction (obtained by extrapolation to zero time)

<sup>5</sup> A preparation of 80 per cent purity as determined by enzymatic reduction was kindly supplied by Dr. A. Kornberg.

was the same at pH 8.6 and 9.0, at pH 8.1, 7.7, and 7.1 it was 81, 57, and 15 per cent, respectively, of the rate obtained at the higher pH values.

Because of the instability of the enzyme protein, it has not been possible, so far, to obtain satisfactory values for the diffusion and sedimentation constants. The turnover number for 100,000 gm. of protein corresponds to a reduction of 6700 moles of DPN per minute at pH 8.6 and 27°. From the data given above, it may be seen that the turnover number at the pH of muscle would be considerably lower. The high concentration of this enzyme in muscle may be related to this relatively low catalytic activity.

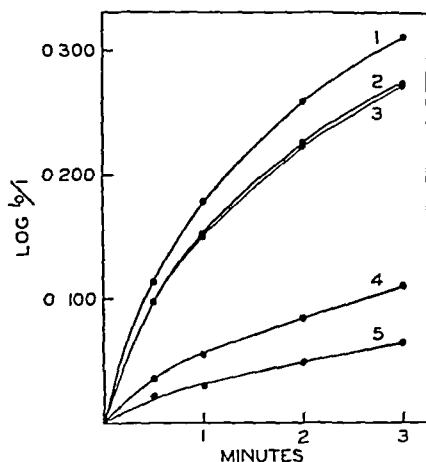


FIG. 6. Rate of inactivation of glyceraldehyde phosphate dehydrogenase by 0.0001 M iodoacetate at 0°. Activity was measured at 27° at pH 8.5. Curves 1 and 2, control samples tested after being kept for 1 and 30 minutes at 0°. Curves 3, 4, and 5, samples tested after being kept for 1, 15, and 30 minutes in the presence of iodoacetate at 0°.

A twice crystallized sample of yeast glyceraldehyde phosphate dehydrogenase, prepared in this laboratory by Dr. E. G. Krebs by a modification of the method of Warburg and Christian, was tested under the same conditions as the muscle enzyme. Dr. Krebs found that the crystalline yeast enzyme requires a reducing agent such as cysteine for full activity, a fact which is not mentioned by Warburg and Christian. The turnover number of the yeast enzyme was of the same order of magnitude as that of the muscle enzyme.

**Iodoacetate.**—The well known inhibition of lactic acid fermentation in muscle and of alcoholic fermentation in yeast has been shown to be at the triose phosphate level (14) when low concentrations of iodoacetate are used. That the triose phosphate dehydrogenase may be classified as an “—SH” enzyme has been established by the work of Rapkine and Trpinac (15–17). It has also been shown that the inhibition by iodoacetate is not instantaneous.



ous and that it cannot be reversed by cysteine. It seemed of interest to repeat some of these observations with the crystalline enzyme preparation.

The curves in Fig. 6 show the rate of inactivation of the enzyme by  $10^{-4}$  M iodoacetate at  $0^{\circ}$  and pH 7.1. No cysteine was used in this experiment and consequently relatively large amounts of enzyme (about 100  $\gamma$ ) had to be used in the activity measurements, which were carried out at  $27^{\circ}$ . A control sample of the enzyme was kept at  $0^{\circ}$  in the absence of iodoacetate in order to determine the amount of spontaneous inactivation.

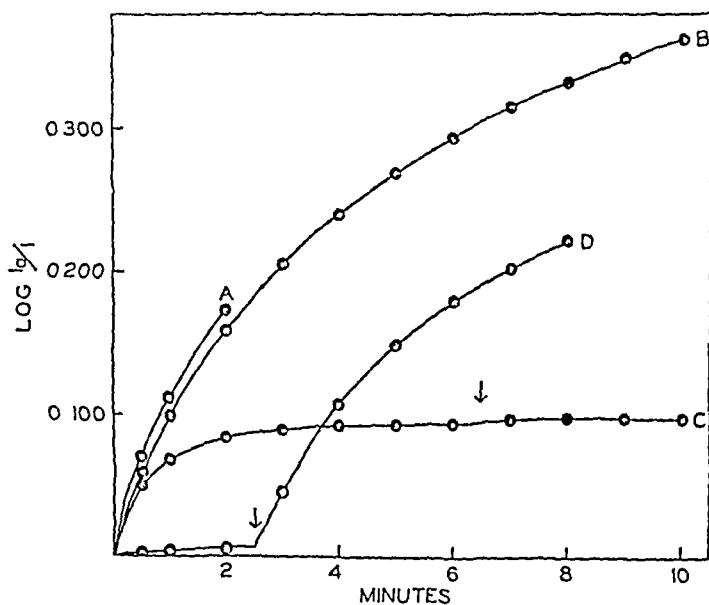


FIG. 7 Irreversibility of the iodoacetate inhibition of glyceraldehyde phosphate dehydrogenase by cysteine. The methods used for obtaining the enzyme in the reduced or oxidized form are given in the text. Curves A and B, activity of the reduced enzyme in the presence and absence of cysteine, respectively. Curve C, activity of the reduced enzyme in the presence of  $0.0004$  M iodoacetate, cysteine added at time indicated by the arrow. Curve D, activity of oxidized form of the enzyme, cysteine added at time indicated by arrow. Activity was measured at  $23^{\circ}$  at pH 8.5.

The enzyme lost 15 per cent of its activity in 30 minutes without iodoacetate and 15, 70, and 85 per cent in the presence of iodoacetate after incubation periods of 1, 15, and 30 minutes.

In the above experiment only a small part of the enzyme was in the active or "reduced" form. This is shown by the fact that the enzyme had only about 10 per cent of the activity it had when tested in the presence of cysteine. The inactive or "oxidized" form of the enzyme does not react with low concentrations of iodoacetate and is converted to the reduced form by cysteine, this simulates a reversal of the iodoacetate inhibition by cysteine.

In Fig 7 are shown the effect of iodoacetate on a fully reduced enzyme and the non-reversibility of the inhibition by cysteine. A solution of 2 mg of enzyme per ml in 0.03 M cysteine-pyrophosphate buffer, pH 8.5, was kept for 15 minutes at 24° in order to reduce the enzyme. This solution was then stored at 0° and aliquots were diluted 30-fold with 0.004 M cysteine-pyrophosphate as needed. As soon as each dilution was made 0.1 ml of the dilution (7 γ of enzyme) was treated as follows. In Curve A, 0.1 ml was incubated for 7 minutes in 0.004 M cysteine-pyrophosphate pH 8.5, and triose phosphate was added to start the reaction, in Curve B, 0.1 ml was added to the otherwise complete, but cysteine-free reaction mixture, the rate of reaction in Curves A and B being nearly the same, in Curve C, 0.1 ml was added to start the reaction in the same way as for Curve B, except that the reaction mixture contained iodoacetate. Curve C shows that even at 23° the inhibition by iodoacetate (final concentration  $4 \times 10^{-4}$  M) requires several minutes for completion and that addition of cysteine, at a time at which the reaction has practically stopped, did not remove the inhibition. In contrast to this is the reactivation by cysteine of the oxidized form of the enzyme, Curve D. In Curve D the enzyme (70 γ per ml) had been allowed to remain at 22° in the absence of cysteine for 30 minutes. The initial rate in the absence of cysteine was insignificant, but the addition of cysteine rapidly reestablished activity.

The authors wish to thank Mr Robert Loeffel and Dr A. A. Green for carrying out the measurements of electrophoretic mobility reported in this paper.

#### SUMMARY

1. The enzyme D-glyceraldehyde-3-phosphate dehydrogenase has been isolated and crystallized by a method involving fractionation with ammoniacal  $(\text{NH}_4)_2\text{SO}_4$  solution of an extract of rabbit muscle prepared with dilute alkali. The enzyme crystallizes from a dilute solution (about 0.1 per cent) of purity level of about 0.5, when the saturation with  $(\text{NH}_4)_2\text{SO}_4$  is 0.72 and the pH 8.2 to 8.4. 1 gm or more of crystalline enzyme is obtained from 500 gm of muscle. On recrystallization from more concentrated enzyme solutions at 0.66 saturation with  $(\text{NH}_4)_2\text{SO}_4$  only a slight gain in specific enzyme activity results.

2. Aldolase and the dehydrogenase can be prepared from the same muscle extract.

3. The recrystallized enzyme is electrophoretically homogeneous over the pH range 6.2 to 7.7. In phosphate buffer, ionic strength 0.1, the isoelectric point is at pH 6.55.

4. Activity of the enzyme was measured spectrophotometrically accord-

ing to the method of Warburg and Christian. In order to obtain maximum activity the enzyme has to be diluted and preincubated in a cysteine (or glutathione) solution.

5. In weak salt solution the enzyme has highest stability around neutrality. At pH 5.2 or 9 it is rapidly denatured even at 0°.

6. The enzyme has one-half maximum activity when the concentration of glyceraldehyde-phosphate is  $5.1 \times 10^{-5}$  moles per liter. The corresponding value of DPN is  $3.9 \times 10^{-5}$  moles per liter.

7. The enzyme activity is highest between pH 8.6 and 9 and drops off sharply on the acid side, so that at pH 7.1 it is only 15 per cent of the maximum rate.

8. Iodoacetate in low concentration ( $10^{-4}$  M) inhibits the enzyme. This inhibition, even at 27°, is not instantaneous. Cysteine does not reverse the inhibition.

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# THE PROSTHETIC GROUP OF CRYSTALLINE D-GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE\*

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It is known that the major portion of the ultraviolet absorption of proteins in the region 250 to 300  $m\mu$  is due to the presence of the aromatic amino acids, chiefly tyrosine and tryptophan. The ultraviolet absorption spectrum of D-glyceraldehyde-3-phosphate dehydrogenase differs appreciably from the spectrum calculated from the chemically determined tyrosine and tryptophan contents (1) both at neutral and alkaline pH, Figs 1 and 2. At neutral pH this difference is particularly noticeable at 250  $m\mu$ . At pH 12 to 13 the absorption maximum of tyrosine plus tryptophan shifts from about 280 to about 290  $m\mu$ . The dehydrogenase exhibits this spectral shift at pH 12 but shows only a barely detectable minimum at 278  $m\mu$ . This property indicates the presence of groups which absorb in the 260 to 280  $m\mu$  region, and which are not grossly affected by changes in pH. Such behavior is shown by the C=N bond as it occurs in purines and pyrimidines. We were therefore led to suspect the presence of nucleotides in the recrystallized protein. This idea was supported by the observation that a considerable amount of humin was formed during acid hydrolysis, indicating the presence of carbohydrate, and that the exhaustively dialyzed enzyme contained 0.126 per cent of phosphorus.

The most likely substance to be looked for was diphosphopyridine nucleotide (DPN), since it participates in the reaction catalyzed by the enzyme. It proved possible to demonstrate that the crystalline enzyme contains a constant amount of DPN in rather firm combination.

The presence of DPN in the enzyme is indicated by the following data:

1. The characteristic absorption maximum of reduced DPN at 340  $m\mu$  appears when glyceraldehyde phosphate and arsenate are added to a 0.77 per cent solution of the enzyme, Fig 3. The absorption observed at 340  $m\mu$  corresponds to 0.6 mole of DPN in 50,000 gm of the dehydrogenase. This band disappears again when pyruvate and lactic dehydrogenase (a purified fraction from rabbit skeletal muscle) are added.

2. The filtrate obtained by precipitating a suitable amount of enzyme

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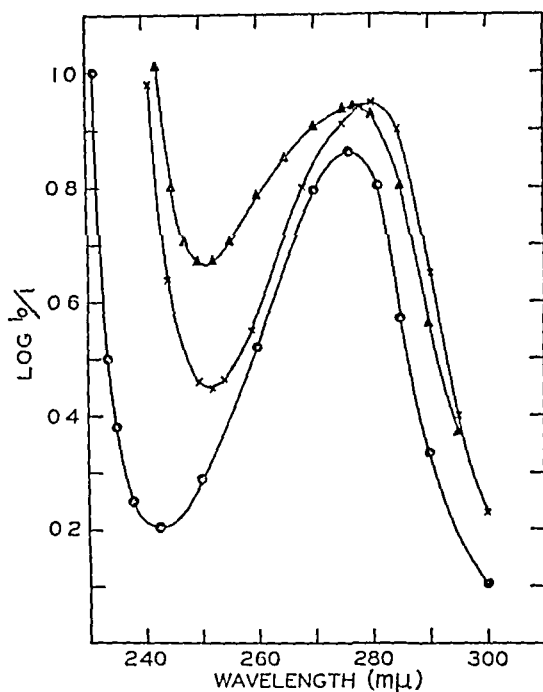


FIG 1 Absorption spectrum of glyceraldehyde phosphate dehydrogenase, 1 mg per ml, at neutral pH  $\Delta$ , untreated enzyme,  $\times$ , enzyme after treatment with phosphatase and precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , 63 per cent of the DPN having been removed (Experiment 3, Table II),  $\circ$ , calculated absorption due to tyrosine and tryptophan present

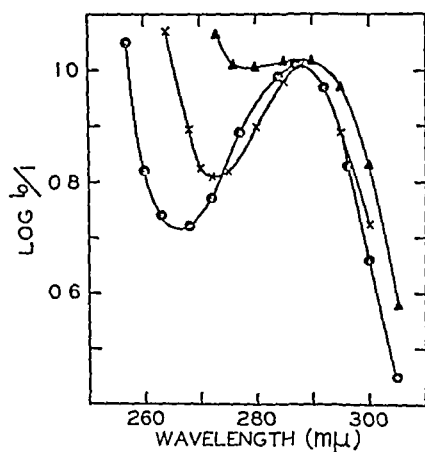


FIG 2 Absorption spectrum of glyceraldehyde phosphate dehydrogenase, 1 mg per ml in 0.1 N NaOH  $\Delta$ , untreated enzyme,  $\times$ , enzyme after treatment with phosphatase and precipitation with  $(\text{NH}_4)_2\text{SO}_4$  (Experiment 3, Table II),  $\circ$ , calculated absorption due to tyrosine and tryptophan present

with trichloroacetic acid can replace DPN in specific enzymatic tests, Fig 4 The absorption observed at 340 mμ after enzymatic reduction of

the trichloroacetic acid filtrate corresponds to 0.8 mole of DPN split off from 50,000 gm of the dehydrogenase

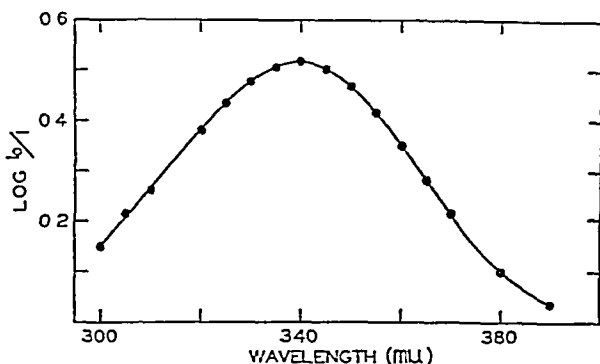


FIG 3 Appearance of the reduced band of DPN at 340  $m\mu$  in a solution containing 7.7 mg of enzyme protein per ml after addition of arsenate and excess glyceraldehyde phosphate. The reference cell contained the same solution with the exception of glyceraldehyde phosphate.

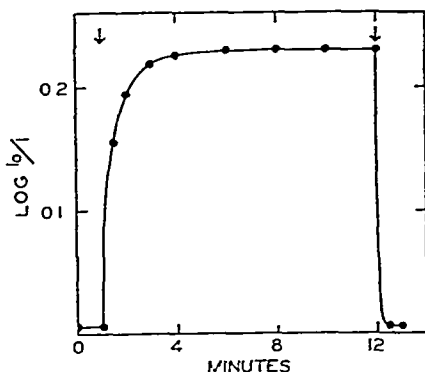


FIG 4 Demonstration of DPN in the trichloroacetic acid filtrate of glyceraldehyde phosphate dehydrogenase. Neutralized filtrate, corresponding to 5.77 mg of enzyme protein, + cysteine pyrophosphate buffer + arsenate + glyceraldehyde phosphate in 3 ml, measured against the control containing neutralized trichloroacetic acid instead of the filtrate. The reaction started at the first arrow by addition of 6  $\gamma$  of glyceraldehyde phosphate dehydrogenase per ml. At the second arrow a solution containing pyruvate + purified lactic dehydrogenase was added to both vessels.

3 The enzyme contains adenine, nicotinamide, ribose, and phosphate in the ratios in which these substances are present in DPN. Table I summarizes the data obtained for the four constituents of DPN.

Adenine and nicotinamide were determined microbiologically.<sup>1</sup> A sam-

<sup>1</sup> We are indebted to Dr. Ethel Ronzoni and Mr. Lester Wicks for the assay of nicotinamide and adenine, respectively.

ple of the exhaustively dialyzed and dried enzyme was hydrolyzed at a bath temperature of 120° for 1 hour in a mixture of formic acid (specific gravity 1.2), concentrated hydrochloric acid, and water in the volume ratios of 5:1:4. This procedure liberates the purines from nucleotides without appreciable decomposition (2). The hydrolysate was repeatedly evaporated to dryness *in vacuo*, then neutralized to pH 6 and diluted to volume.

Assay for adenine was carried out with the adenineless mutant of *Neurospora crassa* 28610 by the procedure described by Mitchell and Houlahan (3). The cultures were grown in 50 ml flasks and the standard curves covered the range of 0 to 200  $\gamma$  of adenine. The growth response indicated the presence of  $0.25 \pm 0.04$  per cent<sup>2</sup> adenine in the enzyme.

TABLE I

*Analysis of Crystalline Glyceraldehyde Phosphate Dehydrogenase for Components of Diphosphopyridine Nucleotide*

Substance	Source	Per mg of enzyme	Substance in 50,000 gm enzyme
		$\gamma$	moles
Adenine	Hydrolysate of dialyzed, dried enzyme	2.5	0.9
Nicotinamide	" " " " "	2.5	1.0
Phosphorus	Dialyzed, dried enzyme, ashed	1.26	2.0
	Trichloroacetic acid filtrate of native enzyme	1.23	2.0
Ribose	Native enzyme	5.90	1.97
	Dialyzed, dried enzyme	5.83	1.95

Nicotinic acid was determined in similar hydrolysates with *Lactobacillus arabinosus* according to the procedure of Snell and Wight (4). This organism was chosen because it shows equal responses to nicotinic acid and nicotinamide, and hence no appreciable error was introduced by incomplete hydrolysis of the amide. The growth response indicated the presence of  $0.25 \pm 0.02$  per cent<sup>2</sup> nicotinamide in the enzyme.

Phosphate was determined by the Fiske and Subbarow method (5). The color was developed in glass-stoppered tubes, graduated at 1 ml, and was read at 660 m $\mu$  in the Beckman photoelectric spectrophotometer, equipped with the microadaptation described by Lowry (6). In this manner 1  $\gamma$  of phosphorus per ml could be determined accurately. Ashing of the trichloroacetic acid filtrates was carried out in the same tubes in which the color was developed.

Ribose was determined by the orcinol reaction (7). Freshly recrystallized orcinol was dissolved in 95 per cent alcohol (50 mg per ml),

<sup>2</sup> Average deviation

this solution is stable for a few days when kept in an amber bottle in the cold. 2 ml of 0.04 per cent ferric chloride in concentrated HCl, 2 ml of unknown (or of standard) and 0.6 ml of the alcoholic or cinol solution were mixed in a Klett colorimeter tube, covered, and heated for 35 minutes at 100°. The peak of absorption of the resulting green color, as determined in the Beckman spectrophotometer, was at 670 m $\mu$ . Readings in this instrument at this wave-length or in the Klett-Summerson colorimeter with Filter 660 gave concordant results.

According to Albaum and Umbreit (8), both free ribose and yeast adenylic acid (3-phosphoriboside) show a much slower rate of color development than does muscle adenylic acid (5-phosphoriboside). The pentose in the enzyme showed the same rate of color development as free ribose, *e g*, the ratio of the densities at 660 m $\mu$  after 8 and 35 minutes of heating was 2.2 for free ribose, 2.15 for the pentose in the enzyme, and 1.29 for muscle adenylic acid. It was therefore of interest to test DPN, although the sample was not pure, it showed a rate of color development similar to that of free ribose<sup>3</sup>. Since pure DPN was not available, free ribose was used as the standard.

Pentose determinations were carried out on 1 to 2 mg of enzyme, either on the protein directly (the solution becomes clear during heating) or on a trichloroacetic acid filtrate. That the DPN which is present in the enzyme appears in the trichloroacetic acid filtrate has been shown in Fig. 4. Pentose determinations on the protein directly and on the trichloroacetic acid filtrate showed that all of the pentose (and phosphate) appeared in the filtrate. A less complete recovery of pentose was obtained in the filtrate of a heat-coagulated enzyme solution.

The average of ten determinations, made on several repeatedly recrystallized enzyme preparations, was  $0.59 \pm 0.04$  per cent<sup>2</sup> of pentose. In these determinations the enzyme crystals suspended in 0.66 saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were centrifuged down at high speed in the cold, and dissolved in water. Aliquots were then used for pentose and for spectrophotometric protein determinations.

As shown in Table I, the same analytical values were obtained on enzyme preparations that had been exhaustively dialyzed against distilled water as on undialyzed preparations. When the enzyme was dialyzed at pH 5.3 or 9.0 overnight at 0°, it lost most of its activity, but retained its DPN as shown by pentose determinations. When a solution of the enzyme was crystallized in the presence of added DPN (about 10 times the amount present in the enzyme) and then recrystallized twice, its pentose content was no higher than that of the original enzyme.

<sup>3</sup> This indicates, incidentally, that the rate of color development is not a reliable criterion for the differentiation of 5- and 3-phosphoribosides.



These experiments show that the DPN is rather firmly combined with the enzyme and that there is a stoichiometric ratio between protein and DPN. The analytical results in Table I indicate that the minimum molecular weight of the protein, based on its DPN content, is 50,000. According to the amino acid data (1), the minimum molecular weight is larger than this by a multiple of 2. Direct spectrophotometric estimation carried out both on the intact enzyme and on a trichloroacetic acid filtrate prepared from it indicates that at least 0.6 mole and 0.8 mole of DPN, respectively, are present in 50,000 gm. of the enzyme protein (Figs 3 and 4).

4. Treatment of the enzyme with phosphatase or norit removes DPN. In both cases the catalytic activity of the enzyme is preserved (Table II),

TABLE II

*Effect of Treatment with Phosphatase or Norit on Pentose Content of Glyceraldehyde Phosphate Dehydrogenase*

Experiment No	Enzyme treated with	Pentose per mg. enzyme		Activity of treated enzyme as per cent of control enzyme
		Control	Treated	
		$\gamma$	$\gamma$	
1	Phosphatase	6.46	1.35	95
2	"	5.64	0.41	67*
3	"	6.31	2.34	92 (96†)
4	Norit	6.50	1.36	86
5	"	6.80	0.65‡	
6	"	6.80	2.43	
			1.09‡	

\* Incubated without cysteine

† After precipitation with  $(\text{NH}_4)_2\text{SO}_4$

‡ After two norit treatments

but the enzyme can no longer be crystallized from 0.66 saturated  $(\text{NH}_4)_2\text{SO}_4$ .

In the phosphatase experiments, solutions of the enzyme (15 to 30 mg per ml.) were incubated 15 to 30 minutes at pH 7.9 to 8.5 at 30° in the presence of 0.1 mg. of phosphatase protein<sup>4</sup> per 25 to 50 mg. of dehydrogenase. In two of the three experiments, 0.006 M cysteine was present during incubation to stabilize the dehydrogenase. Control samples without phosphatase were treated in the same way. At the end of the incubation period, enzymatic activity was determined and the rest of the solution was brought to 0.66 saturation with  $(\text{NH}_4)_2\text{SO}_4$  in a manner designed to effect crystallization of the dehydrogenase. In one instance an amount

<sup>4</sup> We are indebted to Dr. Gerhard Schmidt for the sample of purified intestinal phosphatase.

of phosphatase equivalent to that used during incubation was added to the control sample after salt addition. While crystals formed overnight in the control samples, none formed even after prolonged standing in the samples which had been incubated with phosphatase. Both the crystalline precipitate of the controls and the amorphous precipitate of the experimental samples were separated by centrifugation, washed with 0.7 saturated  $(\text{NH}_4)_2\text{SO}_4$ , and the pentose content of the protein determined. Table II shows that the treated enzyme had lost a considerable part of its pentose, while its enzymatic activity was not destroyed. That DPN was split by the phosphatase was shown by the liberation of inorganic phosphate. In Experiment 3, enzymatic activity was also determined after precipitation with  $(\text{NH}_4)_2\text{SO}_4$ .

In Figs. 1 and 2, absorption spectra of the phosphatase-treated enzyme are presented. The removal of DPN is shown by a marked decrease of absorption between 245 and 265  $\text{m}\mu$  at neutral pH, and between 265 and 285  $\text{m}\mu$  at pH 12. The enzyme used in the absorption measurements was that of Experiment 3, Table II, in which 63 per cent of the DPN had been removed (on the basis of pentose determinations).

In the norit experiments, the enzyme crystals were separated by centrifugation and dissolved in ice-cold water to give a 1 to 3 per cent protein solution. A suspension of norit was added, so that the mixture contained 13 mg. of norit per ml. After gentle agitation for 3 minutes in the cold room the norit was removed by centrifugation and filtration. The loss of protein was 10 to 15 per cent. After treatment with norit, protein was determined by the method of Robinson and Hogden (9). By repetition of the norit treatment it was possible to reduce the pentose content of the enzyme to 0.65  $\gamma$  per mg. The treated enzyme was brought to 0.66 saturation with  $(\text{NH}_4)_2\text{SO}_4$  solution, pH 8.4, one aliquot directly and one after addition of DPN (0.23 micromole per mg. of protein, about 10 times the amount present in the enzyme). In the former case an amorphous precipitate formed and slowly settled. In the latter case, crystals formed rapidly. These crystals were indistinguishable from those of the original enzyme preparation. After two recrystallizations their pentose content was 6.85  $\gamma$  per mg. of protein. This is within the range of values found with untreated enzyme preparations. These experiments show that the enzyme can recombine with DPN and that it crystallizes as a protein-DPN complex. Furthermore, in this recombination the original ratio of protein to DPN is reestablished.

#### SUMMARY

1. Crystalline D-glyceraldehyde-3-phosphate dehydrogenase from rabbit skeletal muscle contains a definite amount of firmly bound diphosphopy-

idine nucleotide (DPN) The minimum molecular weight of the enzyme calculated from its DPN content is 50,000

2 The presence of DPN is shown by the following data (a) The ultra-violet absorption spectrum of the enzyme indicates the presence of groups other than tyrosine or tryptophan in the region 250 to 280  $m\mu$  (b) The addition of D-glyceraldehyde phosphate and arsenate to a concentrated solution of the enzyme leads to the appearance of the characteristic absorption maximum of reduced DPN The band at 340  $m\mu$  disappears when pyruvate and lactic dehydrogenase are added (c) Precipitation by heat or trichloroacetic acid liberates a substance in the filtrate that can replace DPN in dehydrogenase test systems (d) Analyses show that the enzyme contains adenine, nicotinamide, phosphorus, and ribose in the ratio 1 1 2 2

3 Prolonged dialysis of the enzyme between pH 5.3 and 9.0 does not remove the nucleotide The DPN may be removed by treatment with intestinal phosphatase or norit, after such treatment the protein retains its activity but does not crystallize The addition of DPN (after norit treatment) leads to the formation of crystals containing the original ratio of DPN to protein From this it is concluded that the enzyme crystallizes as a protein-DPN complex

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# THE AMINO ACID COMPOSITION OF ALDOLASE AND D-GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE

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Aldolase (1) and D-glyceraldehyde-3-phosphate dehydrogenase (2) catalyze consecutive steps in the series of enzymatic reactions that lead to the formation of lactic acid. These enzymes which constitute about 10 and 7 per cent respectively of the water-soluble proteins of rabbit skeletal muscle show significant differences in solubility, isoelectric point, stability in solution, sulfhydryl sensitivity, and crystal form. The amino acid analyses to be described account, within the limits of error of the methods, for essentially all of the nitrogen in the two proteins. Small but significant differences exist in the proportions of all but a few amino acids and at least four of the amino acids occur in the two proteins with widely differing frequencies. In spite of the differences, there are elements of similarity in the composition of the two enzymes, particularly the low contents of glutamic acid, which differentiate them from the structural proteins of muscle.

The analysis of aldolase is summarized in Table I. Amino acid residue numbers are calculated on the basis of a minimum molecular weight of 140,000. This number is calculated from the molar ratios of the six amino acids present in smallest amounts, as listed in Table II. The result falls between the values 136,000 and 150,000 determined respectively by sedimentation equilibrium and by sedimentation velocity and diffusion on myogen A (3).

Table III contains a summary of the amino acid analyses of the dehydrogenase. In this table the residue numbers are calculated on the basis of a minimum molecular weight of 99,060. The calculations of minimum molecular weight, based upon chemically determined cystine, tyrosine, tryptophan, proline, and phosphorus, and a microbiological methionine determination, are shown in Table IV. The value 99,060 is very close to twice the minimum molecular weight calculated from the content of diphosphopyridine nucleotide (4).

The nitrogen content of the proteins calculated from their amino acid composition is slightly higher than the total nitrogen determined by the micro-Kjeldahl procedure, Tables I and III. However, the differences fall within the limits of possible cumulative error in the summation of twenty independently determined quantities. It is not possible to state with cer-

tainty that the balances as obtained exclude the presence of undetected nitrogenous components, although the presence of such substances appears unlikely

TABLE I

*Amino Acid Composition of Crystallized Aldolase from Rabbit Skeletal Muscle*

Amino acid	Gm per 100 gm protein, average deviations	Gm nitrogen per 100 gm protein	No of residues per 110,000 gm	Methods
Glycine	5.61	1.047	104.7	ID
Alanine	8.56	1.346	134.6	"
Valine	7.40 $\pm$ 0.1	0.885	88.5	LM, LA
Leucine	11.5 $\pm$ 0.2	1.228	122.8	LA
Isoleucine	7.87 $\pm$ 0.4	0.810	84.0	"
Half cystine	1.12 $\pm$ 0.03	0.131	13.1	Chem <sup>1</sup>
Methionine	1.17 $\pm$ 0.2	0.110	11.0	LM, LF
Serine*	6.57 $\pm$ 0.1	0.876	87.6	" Chem <sup>2</sup>
Threonine*	7.1 $\pm$ 0.4			Chem <sup>3</sup>
	6.5 $\pm$ 0.3	0.764	76.4	LF
Arginine	6.33 $\pm$ 0.2	2.037	50.9	LM, Chem <sup>4</sup>
Histidine	4.21 $\pm$ 0.2	1.141	38.0	"
Lysine	9.54 $\pm$ 0.2	1.829	91.4	"
Proline	5.71	0.695	69.4	ID
Phenylalanine	3.06 $\pm$ 0.1	0.260	26.0	LM
Tyrosine	5.31	0.411	41.1	Chem <sup>5</sup>
Tryptophan	2.31	0.317	15.9	Chem <sup>6</sup> 7
Aspartic acid	9.7 $\pm$ 0.2	1.020	102.1	LM
Glutamic "	11.4 $\pm$ 0.2	1.085	108.8	"
Amide nitrogen		0.91	91	Chem <sup>8</sup>
Total	114	16.9		
" nitrogen		16.8		Kjeldahl

Key to methods ID isotope dilution (pipsyl derivative), LM bioassay (*Leuconostoc mesenteroides*), LA bioassay (*Lactobacillus arabinosus*), LF bioassay (*Lactobacillus fermenti*), Chem<sup>1</sup> photometric (phosphotungstic acid), Chem<sup>2</sup> photometric (periodate, chromotropic acid), Chem<sup>3</sup> photometric (periodate, *p*-hydroxydiphenyl), Chem<sup>4</sup> photometric (hypobromite), Chem<sup>5</sup> photometric (mercuric chloride, nitrous acid), Chem<sup>6</sup> photometric (ultraviolet on mercury precipitate), Chem<sup>7</sup> photometric (*p*-dimethylaminobenzaldehyde), Chem<sup>8</sup> titrimetric (micro diffusion)

\* We have not corrected the serine and threonine values for destruction during hydrolysis

Both proteins are relatively high in lysine and have similar but not identical contents of arginine and histidine. Aldolase is higher by about a factor of 2 in glutamic acid and leucine and is considerably lower than the

TABLE II  
Minimum Molecular Weight of Aldolase\*

Amino acid	Molar ratio										
	Gm per 100 gm protein	1	2	3	4	5	6	7	8	9	10
Methionine	1.17	1	2	3	4	5	6	7	8	9	10
Half cysteine	1.12	1.188	2.38	3.56	4.75	5.91	7.13	8.31	9.50	10.7	11.9
Tryptophan	2.31	1.417	2.95	4.31	5.70	7.23	8.68	10.1	11.5	13.0	14.5
Phenylalanine	3.06	2.363	4.72	7.09	9.45	11.8	14.2	16.5	18.9	21.3	23.6
Histidine	4.21	3.455	6.91	10.4	13.8	17.3	20.7	24.2	27.6	31.1	34.5
Tyrosine	5.31	3.373	7.47	11.3	14.9	18.7	22.4	26.0	29.9	33.6	37.4
Mol wt	12,735	25,470	38,205	50,910	63,675	76,410	89,145	100,880	111,615	127,350	140,085
No of in- tegral ra- tios	1	3	2	2	2	2	2	2	3	2	6

\* The complete homogeneity of this protein has not yet been satisfactorily established by physical methods, although the available evidence is favorable (1). For the calculation of a minimum molecular weight of 140,000 to be accepted with confidence, the individual analyses must have an accuracy of  $\pm 1$  per cent. We have not been able to supply independent evidence that the above analyses are actually accurate to within these limits. The above calculations are offered as one test of consistency of the data.

dehydrogenase in its content of valine and phenylalanine. The glutamic acid level in the dehydrogenase is among the lowest ever recorded for a protein and the valine level is among the highest. Serine and threonine are

TABLE III

*Amino Acid Composition of D-Glyceraldehyde Phosphate Dehydrogenase from Rabbit Skeletal Muscle*

Amino acid	Gm per 100 gm protein with average deviations	Gm nitrogen per 100 gm protein	No of residues per 99,100 gm	Methods*
Glycine	6.03 6.15 $\pm$ 0.1	1.125	79.6	ID LM
Alanine	6.72	1.058	74.7	ID
Valine	12.0 $\pm$ 0.1	1.483	101.9	LM, LA
Leucine	6.78 $\pm$ 0.1	0.724	51.2	" "
Isoleucine	9.1 $\pm$ 0.3	0.972	68.7	LA
Half cystine	1.09	0.127	9.0	Chem <sup>1</sup>
Methionine	2.70 $\pm$ 0.2	0.253	18.0	LM, LF
Serine†	6.7 $\pm$ 0.1 7.7	0.893	63.2	" Chem <sup>2</sup>
Threonine†	6.9 $\pm$ 0.2 7.2 $\pm$ 0.5	0.811	57.4	LF Chem <sup>3</sup>
Arginine	5.23 $\pm$ 0.1	1.682	29.8	LM, Chem <sup>4</sup>
Histidine	5.01 $\pm$ 0.1	1.357	32.0	"
Lysine	9.42 $\pm$ 0.3	1.804	63.9	"
Proline	3.67 $\pm$ 0.03	0.446	31.6	ID
Phenylalanine	5.55 $\pm$ 0.06	0.471	33.3	LM
Tyrosine	4.57	0.353	25.0	Chem <sup>5</sup>
Tryptophan	2.05	0.281	9.9	Chem <sup>6, 7</sup>
Aspartic acid	12.4 $\pm$ 0.2	1.304	93.2	LM
Glutamic "	6.8 $\pm$ 0.2	0.647	45.8	LM, LA
Amide nitrogen		0.95	67.2	Chem <sup>8</sup>
Diphosphopyridine nucleotide		0.20	2.0	
Total		16.9		
" nitrogen		16.4		Kjeldahl

\* For the key to the methods, see Table I

† See the footnote, Table I

similar in the two proteins. The cystine contents are similar and low and do not reflect the apparent differences in sulfhydryl sensitivity.

In comparison with the myosin fraction of muscle, data for which have been compiled by Bailey (5), glutamic acid in both enzymes is very low, histidine, serine, threonine, and valine quite high.

TABLE IV  
Minimum Molecular Weight of Crystalline D Glyceralddehyde Phosphate Dehydrogenase from Rabbit Skeletal Muscle\*

Amino acid	Gm per 100 gm. protein	Molar ratio									
		1	2	3	4	5	6	7	8	9	
Half cystine	1 00	2 777	5 55	8 33	11 11	22 85	16 00	19 41	36 0	25 0	
Tyrosine	4 57	1 105	2 21	3 31	4 42	10 25	6 03	7 74	16 1	9 95	
Tryptophan	2 05	1 906	3 992	5 988	7 98	9 98	11 98	13 97	15 97	17 96	
Methionine	2 70	3 510	7 020	10 53	11 04	17 55	21 06	24 57	28 08	31 6	
Proline	3 67	0 447	0 894	1 34	1 79	2 23	2 68	3 13	3 58	4 02	
Phosphorus	0 126	11,007	22,014	33,021	44,028	55 035	66,042	77,050	88,056	99,060	
Mol wt		2	3	2	3	2	3	2	3	5	
No of integral ra- tios											

\* The physical evidence of homogeneity of this protein is not conclusive and we therefore wish to apply here also the comments appended to Table II



## EXPERIMENTAL

The enzymes<sup>1</sup> were dialyzed at 4° first against running tap water, then against frequent changes of distilled water for several days. The contents of the dialysis sacs were then frozen and dried *in vacuo*. After equilibrating with the atmosphere, the moisture contents were determined by drying samples at 0.01 mm pressure over P<sub>2</sub>O<sub>5</sub> at 112°. The ash in the dried enzymes was of the order of 0.1 to 0.2 per cent. Total nitrogen was determined by the micro-Kjeldahl procedure of Miller and Houghton (6). Amide nitrogen was liberated by 2 hour hydrolysis with 2 N hydrochloric acid at 100° and determined by titration after distillation in Conway micro diffusion vessels (7). The latter analyses were carried out on dialyzed solutions of the enzymes analyzed for total nitrogen and corrected for small amounts of residual ammonium salts.

*Lactobacillus arabinosus* 17-5 (No. 8014) was used for the determination of glutamic acid, cystine, isoleucine, leucine, and valine. The basic medium was that of McMahan and Snell (8). It was found that omitting proline and hydroxyproline left a medium supporting normal growth and acid production, and these amino acids were therefore omitted from the media in most of the assays with this organism.

Following the study of the amino acid requirements of *Leuconostoc mesenteroides* P-60 (No. 8042) by Dunn, Shankman, Camien, Frankl, and Rockland (9), this organism was used to check the results obtained with *Lactobacillus arabinosus* and for the determination of the other amino acids essential for its growth. For histidine (10), lysine (11), and glycine (12) the methods described by Dunn and coworkers were used. The basic medium, D, of Dunn *et al.* (9) in double concentration was found suitable for the determination of the other amino acids. When the complete medium was used, addition of the maximum amount of protein hydrolysate employed in an assay caused no further increase in acid production over that given by the basal medium alone. From this it seemed probable that non-specific stimulation effects were absent in the assays of the individual amino acids.

We followed the procedure of analyzing numerous small hydrolysates of independent enzyme preparations. Weighed samples of 20 to 100 mg corrected for moisture were hydrolyzed for 16 hours in 6 N hydrochloric acid at a bath temperature of 125°. The hydrolysates were neutralized to pH 6 and diluted to volume. Growth was measured titrimetrically. The assay curves contained four to six points of a dilution series falling in a sensitive region of the standard curve. An analysis as reported consisted of

<sup>1</sup> These preparations were made by Dr. G. T. Cori and consisted of four to six times recrystallized samples of the enzymes.

three to seven independent assay curves. In Table V are listed the concentration ranges and conditions under which the various assays were carried out. In Table VI are shown the results of some single assay curves obtained on different preparations of the two enzymes.

In the course of the work, four preparations of each enzyme were used and each amino acid was determined in two or more preparations. The observed deviations between different preparations fall within the limits of variation obtained on the same preparation at different times. The average deviation expressed in Tables I and II represents unweighted averages of

TABLE V  
*Summary of Bioassay Conditions*

Amino acid	Configuration of standard amino acid	Organism	Test volume	Range	Incubation time (37°)
			ml	$\gamma$ per tube	hrs
Arginine	L	LM*	5	6-30	48-72
Aspartic	L	"	5	10-60	48-72
Cystine	L	" LA	5	4-20	48-72
Glutamic	L	" "	5	8-80	48-72
Glycine		"	3	8-40	72
Histidine	L	"	3	2-12	72
Isoleucine	DL	" LA	5	5-40	48-72
Leucine	DL, L	" "	5	10-50	48-72
Lysine	L	"	5	10-70	72
Methionine	DL	"	3	2-12	72
Phenylalanine	DL	"	5	4-20	72
Proline	L	"	5	6-60	48
Serine	DL	"	5	6-60	48
Tyrosine	L	"	5	3-15	72
Valine	DL	" LA	5	8-40	48-72
Threonine	DL	LF	3	7-35	48
Methionine	DL	"	3	4-20	48

\* See Table I for an explanation of the contractions

all analyses. Only a few obviously aberrant results were rejected. In general the errors seemed to be largely of a random nature.

Tyrosine, tryptophan, serine, threonine, cystine, arginine, and alanine were determined chemically. The procedure of Lugg (13) with alkaline hydrolysates was followed in essential detail in the determination of tyrosine, except that the colors were read in the Beckman spectrophotometer. The tryptophan mercury complex obtained by Lugg's precipitation procedure was redissolved and determined directly without color development by its absorption in the ultraviolet as suggested by Brand and Sidel (14).

Tryptophan in the dehydrogenase was determined also on the unhydrolyzed protein as described by Sullivan and Hess (15), with results identical with those from the mercury precipitation procedure. It is of considerable interest from the standpoint of protein structure that aldolase by the same direct procedure on the unhydrolyzed protein develops the tryptophan color with *p*-dimethylaminobenzaldehyde much more slowly than other proteins and does not reach the value obtained by the isolation method.

Serine and threonine were determined by periodate oxidation. In the case of serine the formaldehyde produced in the periodate reaction was distilled from the reaction mixture as described by Boyd and Logan (16), but on a somewhat reduced scale, and determined photometrically according to the conditions defined by MacFadyen (17). The acetaldehyde resulting

TABLE VI

*Results of Some Single Amino Acid Assay Curves on Different Preparations of Aldolase and D-Glyceraldehyde Phosphate Dehydrogenase*

Enzyme	Preparation No	Hydrolyzate	Phenylalanine	Glutamic acid	Lysine	Leucine	Valine
Aldolase	1	a	3.17	11.6	8.3	11.6	7.36
		b		11.4			7.54
	2	a	3.06	11.3	8.6	11.8	7.24
		b	3.07		8.9		
	3	a		11.1	8.4	11.3	7.46
Dehydrogenase	1	a	5.51	7.0	9.4	6.56	12.3
		b		6.7	9.2	6.70	11.0
	2	a	5.56		9.8	6.57	11.9
		b	5.45				
	3	a		6.9	9.4	6.71	11.9

from the threonine oxidation was determined by the photometric method of Block and Bolling (18). In these as well as in the other chemical methods that were employed the standard solutions were carried through the same procedures as the unknowns.

Recent investigations of Dunn, Shankman, Camien, and Block (19) with a highly enriched medium show that proline and serine are not essential for *Leuconostoc mesenteroides*. However, using Medium D, we could assay these amino acids with apparent success. The acid production in the blanks was low and there was little increase in the interval between 48 and 72 hours. The data for individual analyses at different levels show good agreement. Microbiological and chemical analyses for serine in aldolase agree to within 3 per cent. The chemical determination of serine in the dehydrogenase yielded a value of 7.69 per cent in contrast to a value of 6.6 per cent obtained by the microbiological procedure. Hydroxylysine is de-

terminated together with serine when the latter amino acid is determined as formaldehyde in the periodate reaction (20). Hydroxyproline has likewise been found to yield formaldehyde under these conditions (21). In contrast, the microbiological assay for serine is certainly independent of hydroxyproline and it is unlikely that it responds to hydroxylysine. Consequently the agreement of chemical and microbiological assays of serine in aldolase is presumptive evidence of the absence of the interfering amino acids in that protein. The possibility of their presence in the dehydrogenase must be left open, since the chemical results were definitely high.

In addition to the determination of threonine by the periodate method, which was rather variable in our hands, we have carried out microbiological assays with *Lactobacillus fermenti*. This organism in a medium deficient only in threonine slowly acquires the ability to grow without it. However, the presence of low concentrations of threonine inhibits adaptation and the organism may therefore be used for assay (22). Our results with *Lactobacillus fermenti* were consistent but were lower than the chemical determinations.

Analyses for cystine, carried out by the polarographic method of Stern, Beach, and Macy (23), established the relative levels but suffered from the uncertainties involved in the required extrapolation to zero protein concentration. The results of microbiological assays with *Leuconostoc mesenteroides* and *Lactobacillus arabinosus* were in essential agreement with the polarographic determinations but showed in individual analyses somewhat larger average variation from the mean than was desired. We therefore also analyzed for total cystine by the photometric method of Kassell and Brand (24). This procedure gave highly reproducible results about 5 per cent higher than those by the above methods.

Difficulties were encountered in the determination of alanine. While this amino acid is not absolutely essential for *Leuconostoc mesenteroides*, *Lactobacillus fermenti*, or *Streptococcus faecalis*, it does have a distinct accelerating effect on growth. Attempts to utilize this effect for assay purposes were without success and we therefore employed the chemical method of Block, Bolling, and Webb ((18) p. 266). This method has serious defects, not the least of which is the fact that it simultaneously determines threonine for which a correction must be applied.

In view of the uncertainties concerning the alanine analyses we submitted samples of the proteins to Dr. Sidney Udenfriend, of the Department of Chemistry, New York University Medical School, who kindly undertook analyses by the isotope dilution procedure of Keston, Udenfriend, and Cannan (25). He found 8.56 per cent alanine in aldolase and 6.73 per cent in the dehydrogenase, in contrast to our values of 7.87 and 5.92 per cent respectively. Since the analyses with radioactive *p*-iodophenylsulfonyl

chloride were controlled with respect to recovery from artificial amino acid mixtures and would have been low rather than high if partial racemization had occurred during hydrolysis, we accept them in preference to our own

In addition to the analysis for alanine, Dr Udenfriend also carried out analyses for proline and glycine. The proline results were about 9 per cent lower than the microbiological assays and, since they were performed with

TABLE VII  
*Amino Acid Analyses of Crystallized Bovine Serum Albumin*

Amino acid	Previous work		Present work	
	Gm per 100 gm protein	Method*	Gm per 100 gm protein	Method†
Glycine	1.96	<i>a</i>	2.0	LM
Valine	6.5	<i>b</i>	6.6	LA, LM
Leucine	13.7	"	13.2	LM
Isoleucine	2.9	"	2.7	"
Cystine	6.52	"	6.50	Chem <sup>1</sup>
			6.2	LM
Methionine	0.81	"	0.80	"
Serine	4.5	"	4.9†	"
Threonine	6.5	"	7.1†	Chem <sup>3</sup>
Arginine	6.2	"	5.9	LM
Histidine	3.80	"	4.00	"
Lysine	12.42	<i>a</i>	11.9	"
Phenylalanine	6.2	<i>b</i>	6.05	"
Tyrosine	5.53	<i>a</i>	5.50	Chem <sup>5</sup>
Tryptophan	0.58	<i>b</i>	0.58	Chem <sup>7</sup>
Proline	5.6	"	5.5	LM
Aspartic acid	10.25	<i>a</i>	10.45	"
Glutamic "	16.95	"	17.0	LA

\* Method *a*, Shemin (28) Method *b*, Brand (26)

† For the key to the methods used, see Table I

‡ In order to facilitate the comparison with Brand's figures we have followed his procedure here of applying an arbitrary 10 per cent correction for the destruction of serine and threonine during the hydrolysis

the DL-proline derivative as carrier, a second analysis of the dehydrogenase was made with the L-proline derivative as carrier. No significant difference was observed in the results. The discrepancy may be due in part to impurities in the standard. Although the L-proline sample employed exhibited the proper optical rotation and theoretical nitrogen content, it liberated about 0.2 per cent primary amino nitrogen in the manometric Van Slyke apparatus. The control analyses compared favorably with the previously published figures also obtained microbiologically (Tables VII

and VIII) A discrepancy of this type suggests the possibility that partial destruction during hydrolysis, which is difficult to establish, will undoubtedly lead to low results in isotope dilution analysis but may yield products which are metabolically active in microbiological assay The isotopic glycine results were aldolase 5.61 per cent and dehydrogenase 6.88 per cent, as compared with microbiological values of 6.12 and 6.98 per cent respectively

TABLE VIII  
*Amino Acid Analyses of Zinc Insulin*

Amino acid	Previous work* gm per 100 gm protein	Present work	
		Gm per 100 gm protein	Method†
Glycine	4.6	4.5	LM
Valine	8.8	9.1	"
Leucine	13.4	13.0	"
Isoleucine	2.9	2.8	LA
Cystine	11.6	11.7	Chem <sup>1</sup>
Serine	5.8	6.6†	LM
Threonine	3.16	3.5†	Chem <sup>3</sup>
Arginine	3.47	3.4	LM
Histidine	5.28	5.30	"
Lysine	2.6	2.4	"
Phenylalanine	7.9	7.95	"
Tyrosine	12.3	12.2	Chem <sup>5</sup>
Glutamic acid	20.2	19.9	LA
Proline	2.9	2.9	LM

\* Brand ((26) p. 198)

† For the key to the methods used see Table I

‡ See the foot note to Table VII

Because the glutamic acid levels are of considerable interest, we employed, in addition to *Leuconostoc mesenteroides* and *Lactobacillus arabinosus*, S. R. strain 12 of *Clostridium welchii* which Gale has shown to decarboxylate specifically L-glutamic acid in protein hydrolysates (27). Our experiments with this method have not been extensive but the results tend to confirm the previously determined figures.

During the process of standardizing our methods, we carried out analyses of crystallized bovine serum albumin, No. 46, from Armour and Company, and crystallized zinc insulin, 24 activity units per mg, from Eli Lilly and Company. Different and possibly more highly purified samples of these proteins had previously been subjected to intensive analysis by Brand and coworkers by chemical and microbiological procedures (26). Isotope dilu-

tion analyses of bovine serum albumin have been performed by Shemin (28)

The control analyses agree rather well with the previously published work (Tables VII and VIII) Early analyses for isoleucine were very much higher than Brand's figures and we were led to suspect the presence of isomeric impurities in our standard DL-isoleucine Subsequent assays with a DL-isoleucine standard that had been shown by Dr M S Dunn to be about 97 per cent pure by solubility studies indicated that our original sample contained about 19 per cent impurity, presumably the *allo* isomer Assays with the new DL-isoleucine standard, as shown in Tables VII and VIII, are now slightly lower than the values of Brand

The discrepancies in the serine and threonine values find no ready explanation and seem to be due in part to defects in the methods, some of which have been discussed previously Although it has not yet been possible to evaluate all of the methods on an absolute basis, the control analyses speak well for their reproducibility in different hands

We are indebted to Dr M S Dunn and coworkers for helpful advice and for samples of glycine media and of L-leucine and DL-isoleucine of known purity

#### SUMMARY

1 Aldolase and D-glyceraldehyde phosphate dehydrogenase have been subjected to complete amino acid analysis by microbiological and chemical procedures

2 Within the limits of errors of the methods, all of the nitrogen of aldolase has been accounted for by its content of eighteen amino acids

3 The nitrogen of D-glyceraldehyde phosphate dehydrogenase is likewise accounted for by its content of eighteen amino acids and diphosphopyridine nucleotide

4 The minimum molecular weight of the dehydrogenase calculated from five observed residue numbers is 99,000 and that of aldolase is 140,000

5 Analyses of crystallized bovine serum albumin and of crystallized zinc insulin are presented These analyses for the most part are in agreement with previously published figures

6 Included in the results are isotope dilution analyses for glycine, proline, and alanine by Dr Sidney Udenfriend of the Department of Chemistry, New York University College of Medicine

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# THE METABOLISM OF *p*-AMINOSALICYLIC ACID IN THE ORGANISM OF THE RABBIT

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The importance of *p*-aminosalicylic acid in the treatment of tuberculosis has been demonstrated both clinically and experimentally (1-6). The close structural relationships between benzoic, salicylic, *p*-aminobenzoic (PABA), and *p*-aminosalicylic (PASA) acid warrant a comparative study of the metabolism of these substances in the animal body.<sup>1</sup> Recent workers, interested in the behavior of PABA in man, the dog, and the rabbit, have concerned themselves chiefly with the acetylation of this compound, with little consideration for other possible metabolic reactions such as conjugation with glycine to form the conjugate, *p*-aminohippuric acid. It is of interest to note that *in vitro* synthesis of this latter compound from PABA by homogenates and tissue liver slices has been demonstrated recently (8-10). In a comprehensive study of the fate of salicylic acid in man (11), 80 per cent of a single dose was excreted by the kidneys, of this 20 per cent was unchanged, 55 and 25 per cent in conjugation with glycine (as salicyluric acid) and glucuronic acid respectively, and a small amount (4 to 8 per cent) as gentisic acid and other products of oxidation. In children and in febrile patients, oxidation of salicylic acid was increased, while, in fever, the conjugation to form salicyluric acid was diminished. Lutwak-Mann (12) isolated gentisic acid from the urine of rats to which salicylic and acetylsalicylic acids had been fed, and observed that when the animals were poisoned with carbon tetrachloride or yellow phosphorus the formation of gentisic acid was decreased. Detailed studies of the fate of salicylic acid in the rabbit are not available. In general, however, as pointed out by Quick (7), the conjugation of glycine with an aromatic carboxyl group is markedly inhibited by a substituent group in the ortho position as in salicylic acid. Systematic studies of the metabolism of salicylic acid and related compounds with more specific methods for the determination of products of metabolism are needed. It has seemed,

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<sup>1</sup> For a review of older work on the metabolism of substituted benzoic acids, the studies of Quick (7) should be consulted

therefore, desirable to have information as to the biological behavior of PASA in as many animals as possible in view of the marked species variations known with other aromatic acids, *e g* , benzoic acid in man, the dog, or the rabbit. As an initial contribution to this problem, we present here the results of an investigation of the metabolism of PASA in the rabbit.

#### EXPERIMENTAL

*Collection of Specimens*—Male rabbits, 2 to 4 kilos in weight, were maintained on a diet of cabbage and oats. The procedures of caging and the collection of the urine were those usually employed in this laboratory (13). Decomposition was prevented by the addition of 10 ml. of 1 per cent nitric acid to the collection bottle. The PASA was administered orally through a stomach tube either as a solution of the sodium salt or as the acid in fine suspension in water, or was injected intramuscularly as a freshly prepared solution of the sodium salt.

*Compounds Fed, Stability*—The PASA was crystallized from absolute alcohol and dried over phosphorus pentoxide. The crystalline white material melted at 150° (decomposition) and contained 9.09 per cent of nitrogen (theoretical, 9.15 per cent) on analysis (semimicro-Kjeldahl). A 10 mg. per cent solution of the acid had an initial pH of 3.2, which changed to 4.2 on standing at room temperature for 48 hours and to 4.7 in a week, after being heated on the steam bath for 1 hour, the pH of the solution was 5.4. This change in reaction is believed to be due to a slow decarboxylation of the compound in aqueous solution with the formation of *m*-aminophenol. As shown by the non reaction (to be discussed subsequently), the decarboxylations in 24 and 48 hours were 41 and 54 per cent respectively. When the material was dissolved in 4 per cent toluenesulfonic acid (pH 1.0), only 18 per cent decarboxylation occurred at room temperature in 24 hours, although this reaction was complete after heating the solution on the steam bath for 1 hour.

Crystallized PASA was suspended in 95 per cent alcohol and the mixture was carefully neutralized with alcoholic sodium hydroxide until a definite pink color with phenolphthalein was obtained. This solution of sodium salt was poured slowly into cold ether, with shaking. The precipitated sodium salt was filtered, washed with cold ether, and dried. The salt appeared to form a loose molecular compound with ether but the solvent could be removed by continuous evacuation at 0.5 mm. pressure over phosphorus pentoxide until the weight became constant. A sample thus dried gave a loss of 14.31 per cent. The dried sample contained 7.48 per cent of nitrogen (theoretical 7.11 per cent). In all cases in which the sodium salt was administered orally to the rabbits, the amount administered is calculated as the dried salt. The sodium salt was very soluble

in water. A 100 mg per cent aqueous solution of the sodium salt (pH approximately 6.8) appeared to be stable even at a pH of 9.0 and no decarboxylation (as was evidenced by the iron reaction) occurred when the solution was heated on the steam bath for 1 hour. There was slight discoloration in concentrated solutions on exposure to light, but samples of the dry sodium salt have been kept for several months without deterioration.

*Methods of Analysis*—Two color reactions have been employed to follow the metabolism of PASA. Compounds containing the orthohydroxybenzoyl group give an amethyst color with ferric salts in an acid medium, due to the formation of an iron complex. The quantitative aspect of this test has been the subject of a careful study by Nicholls (14) and Brodie and coworkers (15). In PASA, the color formation should not take place if the OH group has been blocked by substitution or if decarboxylation of the COOH group has occurred. 2-Hydroxy-4-aminohippuric acid (the PASA analogue of salicylic acid) also gives a similar color, but of different intensity.

The free arylamino group is characterized by the lemon yellow color formed with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent). This reaction has been applied to the determination of sulfanilamide derivatives in plasma and urine (16-19). Morris (20) has shown that, for the successful application of the *p*-dimethylaminobenzaldehyde reaction to the estimation of sulfanilamides, the test must be carried out in acid media (pH 1.5 to 1.7) and in the presence of an excess of the aldehyde reagent. Both of these conditions are met in our procedures by carrying out the reaction at a pH of 2.0 to 2.2 and adding a constant amount of Ehrlich's reagent buffered with a mixture of sodium acetate and acetic acid. N-Acetylated PASA did not give this reaction and could be estimated after hydrolysis with acid as *m*-aminophenol.

*Reagents*—

1. 20 per cent *p*-toluenesulfonic acid (TSA). 200 gm of TSA monohydrate (21, 22) were dissolved in 600 ml of water, stirred with a small amount of norit K for 10 minutes, filtered, and the filtrate made up to 1 liter. 2 volumes of this stock solution were diluted with 3 volumes of water to give 8 per cent TSA.

2. Iron reagent. A 1.68 per cent solution of  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (Merck) in 0.07 N nitric acid.

3. Ehrlich's reagent. 2 gm of *p*-dimethylaminobenzaldehyde (Eastman) were dissolved in 100 ml of glacial acetic acid and added to an equal volume of a 4 M solution of anhydrous sodium acetate. When 1 ml of this reagent was added to 10 ml of 4 per cent TSA, the pH was 2.0 to 2.2.

*Measurements of Color*—All measurements of color intensity were made

with a Coleman Universal spectrophotometer (model 11) The blank was set at 100 per cent transmission and matched tubes were used for containers

*Standard K Values*—A sample of PASA, twice crystallized from absolute alcohol and dried over phosphorus pentoxide, was used to prepare a standard stock solution containing 1 mg per ml Since PASA is not freely soluble in water and is stable in aqueous solution only as the sodium salt, the acid (100 mg) was neutralized with 1 per cent sodium carbonate solution and made up to 100 ml For the iron reaction, a 10-fold dilution of the stock solution was made 1 to 8 ml samples were added to tubes containing 2 ml of 20 per cent TSA and the volume was made up to 10 ml, so that the final concentration of TSA was 4 per cent 2 ml of the iron reagent were added to each tube and the color was read at 500  $m\mu$  with a water blank similarly treated There seemed to be no delay in the development of the color, nor any fading within a reasonable length of time However, the readings were taken between 5 and 20 minutes The average *K* value for the concentrations of from 0.1 to 0.8 mg was used for the calculations of the unknowns

For the Ehrlich reaction, the stock solution was so diluted that 1 ml of the diluted solution contained 0.005 mg of PASA The procedure as outlined above was followed, except that 1 ml of Ehrlich's reagent was used to develop the color, which was read at 450  $m\mu$  Here also the color developed immediately and was quite stable, readings were usually taken between 5 and 20 minutes The average *K* value obtained over the concentration range of 0.005 to 0.040 mg was used for calculations

Similarly the average *K* value (Ehrlich) for *m*-aminophenol was obtained for a range of 0.005 to 0.040 mg to calculate the *m*-aminophenol (MAP) produced after hydrolysis As would be anticipated, this value agrees very well with that obtained directly from known amounts of PASA after hydrolysis in 4 per cent TSA

*Iron Reaction*—To 5 ml of urine so diluted as to contain 0.2 to 1.2 mg of PASA, 5 ml of 8 per cent TSA were added, followed by 2 ml of the iron reagent The pink color was read at 500  $m\mu$  with a similarly treated normal urine blank for 100 per cent transmission

*Ehrlich's Reaction*—(a) The free arylamino group was estimated by adding 5 ml of 8 per cent TSA to 5 ml of a suitably diluted urine sample, followed by 1 ml of Ehrlich's reagent The color was read at 450  $m\mu$  with a similarly treated normal urine blank for 100 per cent transmission (b) For the determination of total arylamino groups, 5 ml of the suitably diluted urine were hydrolyzed with 5 ml of 8 per cent TSA in a loosely stoppered graduated tube in a boiling water bath for 1 hour The tube was then cooled, the level of the fluid again brought to the mark as necessary, and the color developed with 1 ml of Ehrlich's reagent A similarly

treated normal urine was used as the blank and the color was read at 450  $m\mu$ . Since PASA, under the above conditions, is completely decarboxylated to MAP, the results calculated as MAP were converted into PASA by multiplying by the factor 153/109. In the above estimations, the 24 hour sample of urine previous to the administration of PASA was suitably diluted to give suitable blanks to correspond to 6 and 18 hour specimens of normal urine.

Glucuronic acid was estimated by the modification of the Maughan, Evelyn, and Browne method (13) commonly used in this laboratory, and creatinine and urinary sulfur partition by the methods of Folin. PASA did not interfere with the estimations of glucuronic acid or creatinine.

#### DISCUSSION

150 mg daily of PASA (as the sodium salt) per kilo of body weight could be administered orally to male rats for 2 weeks without signs of toxicity or impairment of growth. To determine whether any significant fraction of the dosage escaped absorption, the feces were analyzed for PASA in two experiments with rabbits. The amount found was so small, less than 5 mg in a 24 hour feces, that it was thought that the routine analysis of the feces was not necessary.

In Table I, the results of urinary excretion of PASA and derivatives based on the Ehrlich reaction are summarized. 53 to 80 per cent of the compound ingested orally could be accounted for in the urine. On the other hand, when the compound was administered by intramuscular injection, there was a greater recovery, about 90 per cent, as indicated by the Ehrlich reaction. In either case, 70 to 90 per cent of the total amount of PASA recovered was excreted in the first 6 hours, and during this period 55 to 80 per cent of the amount excreted was in the acetylated form. The lower levels of acetylation, about 40 per cent in the 6 hour period, when the compound was administered parenterally, may be due to the rapidity of excretion. The rapid elimination of PASA makes it improbable that the fraction not recovered could be stored in the tissues. It has been believed that a certain amount of salicylic acid may be destroyed in the organism (23). This is in contrast to the unsubstituted compound, benzoic acid, which may be almost completely accounted for in the urine after administration.

Urine was collected from four rabbits, each fed 1 gm of PASA on 2 successive days. The sample was treated with neutral lead acetate. The precipitate was centrifuged and the centrifugate was made just alkaline with ammonium hydroxide and treated with basic lead acetate. The precipitate was centrifuged and was decomposed by treatment with hydrogen sulfide. The lead sulfide was filtered, washed, and the filtrate and the

washings, after aeration, were kept in the refrigerator for 72 hours. The crystalline white solid (400 mg) was filtered, washed with a small quantity of ice-cold water, and dried in the desiccator. The acetyl PASA thus obtained melted at 212° with decomposition. The mother liquor was extracted four times with 200 ml portions of ether and from the ether extract 1 gm of an impure white solid was obtained. On washing this with small quantities of ether, 150 mg of the sparingly soluble acetyl compound remained on the filter. The ether-soluble fraction (800 mg) after crystalli-

TABLE I

*Urinary Excretion of PASA and Derivatives by Rabbits (Ehrlich Reaction)*

The sodium salt used for oral administration was prepared as described in the text. For injection, the sodium salt was prepared freshly by the neutralization of the PASA with the theoretical amount of sodium bicarbonate.

PASA administered	Method of administration	PASA excreted				PASA acetylated, of total excreted	
		6 hrs		24 hrs		6 hrs	24 hrs
		Unchanged	Acetylated	Unchanged	Acetylated		
		per cent of intake	per cent of intake	per cent of intake	per cent of intake	per cent	per cent
mg							
397	Salt, oral	21	42	22	58	67	72
397	" "	25	34	26	48	58	65
502	" "	16	45	18	62	74	77
362	" "	11	27	13	40	70	75
330	" "	9	39	10	53	82	84
571	Acid, "	20	25	25	47	56	65
796	" "	14	27	17	36	65	68
750	" "	12	22	17	42	65	71
580	Salt, intramuscular	46	28	48	39	38	45
740	" "	47	32	49	42	40	46

zation from absolute alcohol was identified as PASA. The acetyl compound was purified by solution in dilute sodium bicarbonate solution (charcoal) and precipitation with dilute hydrochloric acid. The sample thus obtained was filtered, washed with cold water, dried first in a desiccator, and finally in an oven at 60°. The isolated compound and a pure sample of acetyl PASA were heated in a bath whose temperature was rising slowly. They melted at 212° and 214° respectively with decomposition.<sup>2</sup>

<sup>2</sup> According to a private communication, Mr. Leonard Doub has observed a decomposition point of 233-234° (uncorrected) in a bath when the temperature was increased rapidly. The decomposition point was not precise and depended on the rate of heating.

A mixed sample of the two showed no depression in the melting point. Found, nitrogen 7.23 per cent, theory, nitrogen 7.19 per cent. The isolated acetyl derivative was also analyzed by the color reaction with the iron reagent to give 98.5 per cent purity and after hydrolysis with 4 per cent TSA for 1 hour under the usual conditions, as *m*-aminophenol, 98 per cent, by the Ehrlich reaction.

Since in most experiments the PASA was administered orally, an attempt was made to demonstrate whether the contents of the stomach or the intestines would decompose the compound *in vitro*. The stomach and the small intestines of rats and rabbits were removed separately and the

TABLE II

*Incubation of PASA with Homogenized Gastric and Intestinal Contents of Rabbit and Rat*

In all experiments except Experiment 19.9, the contents were incubated with finely divided PASA, in Experiment 19.9 the acid was dissolved in 4 ml. of 5 per cent sodium bicarbonate.

Experiment No	Organ	Time of incubation	PASA	PASA recovered			
				Iron reaction	Ehrlich		
					Free (a)	Total (b)	Conjugated (b - a)
		min	mg	per cent	per cent	per cent	per cent
19.9 (rabbit)	Stomach	90	104	97	84	98	14
	Intestine	210	101	103	80	98	18
20-10 (rabbit)	Stomach	90	108	96	70	92	22
	Intestine	210	101	97	79	96	17
21-11 (rat)	Stomach	90	32	94	81	91	10
	Intestine	210	50	92	86	94	8

contents were homogenized with water in a Waring blender. PASA or its sodium salt was incubated with the emulsion for a definite period, and the mixture was then treated with a sufficient quantity of 20 per cent TSA to make a final concentration of 4 per cent TSA and filtered. The filtrate, centrifuged as necessary, was analyzed by the iron and the Ehrlich reactions. The results were also checked by estimating by the Ehrlich reaction the *m*-aminophenol formed after heating the filtrate for 1 hour on the steam bath. All necessary dilutions were made in 4 per cent TSA. A control experiment was run in each case without PASA, which served as a blank in the estimations. The results are summarized in Table II.

One might have expected the decarboxylation of PASA, associated with the acidity available in the stomach and the activity of the intestinal flora



However, the results of the incubation of PASA with gastric and intestinal contents of the rabbit and the rat definitely indicated that there was no decarboxylation *in vitro*, since essentially all the material originally added was recovered by the non reaction. The only change detected was a small amount of acetylation, 14 to 23 per cent in the case of the rabbit and 6 to 10 per cent with the rat. This needs further investigation.

The earlier experiments indicated conjugation with acetic acid to give the N-acetyl derivative. However, PASA might be conjugated in the animal body in more than one way, as there are three groups in the molecule which might be used in such reactions. Owing to the ease with which the compound is decarboxylated in the test-tube, it might have been expected that this reaction would take place in the animal body. If this were the case to any considerable extent, the resulting *m*-aminophenol should be acetylated or possibly conjugated with glucuronic acid or sulfuric acid (24). One might also observe the introduction of a hydroxyl group in the molecule of PASA *in vivo*, either by direct oxidation of the compound and its acetyl derivative or by a formation and rearrangement of the hydroxy-amino derivative,<sup>3</sup> to form 2,5-dihydroxy-4-amino- or acetylaminobenzoic acid. These compounds might be conjugated with glucuronic or sulfuric acid, particularly at the hydroxyl group in the 5 position.

Studies of urinary sulfur after feeding PASA indicated practically no increase in ethereal sulfate. No uniformity is displayed in the excretion of extra glucuronic acid after the administration of PASA. From experience in this laboratory, we consider that, with rabbits, any value for extra glucuronic acid less than 70 mg. either in the 6 hour or 18 hour period is of little significance. In only two of ten experiments was the extra glucuronic acid in the 6 hour period, the period of rapid excretion of PASA, greater than 70 mg., values of 75 and 103 mg. being obtained. On this basis we are inclined to believe that the amounts of extra glucuronic acid after the administration of PASA orally or parenterally are not indicative of any significant conjugation.

It may, therefore, be assumed that there is neither considerable decarboxylation of PASA nor introduction of a new hydroxyl group *in vivo*, and the amounts of free and combined derivatives in the urine as estimated by the Ehrlich reaction represent primarily free and acetylated PASA.

The question of the conjugation of glycine with the carboxyl group of PASA may be discussed. 2-Hydroxy-4-aminohippuric acid gives a color with the non reagent similar to those with PASA and acetyl PASA, however, it is not decomposed completely to *m*-aminophenol under the conditions used for hydrolysis, but still gives considerable color with the non

<sup>3</sup> Williams (25) has discussed the possible formation of *p*-hydroxylaminobenzene-sulfonamide in metabolism. A number of hydroxy derivatives of the sulfonamides have been isolated and characterized in studies both *in vivo* and *in vitro* (25-27).

reagent after such a procedure. The experimental urine did not give any color with the iron reagent after hydrolysis, indicating the absence of the glycine conjugate. This is in accordance with the belief that there is little or no conjugation of glycine with ortho substituted benzoic acids with the exception of *o*-chlorobenzoic acid (7). It may be noted, however, that a considerable proportion of salicylic acid, an orthohydroxyl derivative of benzoic acid, when fed to man, is reported to be excreted in conjugation with glycine (11).

The color observed in the iron reaction with the experimental urine should be due to the presence of both the free and acetylated PASA. It may be pointed out that, since the two compounds give different intensities of color with the iron reagent, the two *K* values being 0.524 and 0.264 respectively under our experimental conditions, the evaluation of the observed optical activity in terms of PASA alone might suggest considerable decarboxylation of the compound *in vivo*. Therefore, the values for PASA and acetylated PASA obtained by the Ehrlich reaction with the experimental urines have been used to calculate the expected individual optical densities in the iron reaction. The difference between the *observed* optical density and the sum of the *calculated* optical densities of the two forms of PASA present might well be due to the presence of small amounts of other metabolites.

The presence of *m*-aminophenol and its *N*-acetyl derivative, which do not react with the iron reagent, would give negative values in such a calculation, while the presence of  $\beta$ -resorcylic acid, which reacts with the iron reagent, would give positive values. The difference ("unknown") has been somewhat arbitrarily expressed as PASA for the calculation of the net results of the iron reaction (Column 3). The values calculated in this manner are presented in Table III.

It may be noted that the amounts of unknown metabolite thus calculated as differences represent a relatively small proportion of the total PASA (free and acetylated) excreted and that the values are both positive and negative. The data fail to offer any significant evidence of decarboxylation of PASA in the organism of the rabbit. This would be shown by consistent negative values. On the contrary, the differences, thus calculated for the 6 hour period, the period in which maximal excretion of the PASA is occurring, are only slightly positive in six of the ten experiments recorded and none of the negative values represents any large proportion of the total amount excreted.

The algebraic sum of the amounts in Columns 1, 2, and 3 represents the total amount of PASA or derivatives excreted, as indicated by the iron reaction. The per cent of the total PASA administered which is represented by this value is calculated in Column 4. This figure may be compared with the total PASA, excreted in the 6 hour period as obtained by

the Ehrlich reaction, which is shown in Column 5. The average total recoveries for the eight feeding experiments as calculated by the two methods are 45.3 and 48.6 per cent respectively. The differences between these average values are not significant.

We are indebted to Mr. Leonard Doub of Parke, Davis and Company for generous gifts of *p*-aminosalicylic acid and some of its derivatives and for the analysis of the sodium salt of PASA, and to Dr. A. C. Bratton, Jr., for suggestions relating to the colorimetric methods of analysis used in

TABLE III

*Interpretation of Iron Reaction in Terms of Excretions of PASA and Derivatives As Determined by Ehrlich Reaction*

The calculations are for the 6 hour period immediately after the administration of PASA. The "unknown" metabolites (Column 3) are expressed in terms of PASA. Column 5 is included for comparison. For explanation of the calculation, the text should be consulted. The PASA administered can be obtained from the corresponding data of Table I.

Free (1)	Acetyl (2)	Unknown (3)	Sum of intake (4)	Ehrlich reaction of intake (5)
<i>mg</i>	<i>mg</i>	<i>mg</i>	<i>per cent</i>	<i>per cent</i>
82	165	-42	52	63
98	136	-27	52	59
82	228	-34	55	61
41	98	+9	41	38
29	129	-29	39	48
112	145	+13	47	45
113	214	+5	42	41
88	162	+4	34	34
268	165	+17	78	74
351	239	+87	91	79

this study. In particular the interest and helpful criticism of Dr. Bratton are gratefully acknowledged.

## SUMMARY

1. An aqueous solution of *p*-aminosalicylic acid (PASA) undergoes a gradual decarboxylation at room temperature with the formation of *m*-aminophenol. This is evidenced by the change in pH toward the alkaline side as well as by the decrease in the intensity of the reaction with ferric salts. The sodium salt, however, forms a stable solution when dissolved in water.

2. The metabolic fate of PASA in the rabbit has been studied. PASA

is rapidly excreted in the urine in the unchanged and acetylated forms. From 40 to 60 per cent of the amount administered orally could be accounted for in 24 hours as the acetyl derivative. Both free and acetylated PASA have been isolated from the experimental urines.

3 PASA is absorbed almost completely from the gastrointestinal tract and there is no indication of decarboxylation *in vitro* in the presence of gastric or intestinal contents. No clear-cut evidence of any appreciable decarboxylation *in vivo* has been obtained.

4 Data obtained for extra glucuronic acid do not suggest any significant conjugation as a glucuronide, while the possibility of conjugation with glycine appears remote. No increase in ethereal sulfate formation is indicated.

5 The chief mechanism of detoxication of PASA in the rabbit appears to be through acetylation.

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# PASSAGE OF SELENIUM THROUGH THE MAMMARY GLANDS OF THE WHITE RAT AND THE DISTRIBUTION OF SELENIUM IN THE MILK PROTEINS AFTER SUBCUTANEOUS INJECTION OF SODIUM SELENATE

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One of the pathways by which selenium may be eliminated from the animal body other than through the kidney, gastrointestinal tract, and lung is through the mammary glands into the milk of lactating animals. This has been shown by several investigators to take place in certain domestic animals. Dudley (1) found 0.02 to 3.00 parts per million of selenium in the milk of selenized cows. Smith *et al.* (2) reported in an extensive survey that milk from cows grazing in seleniferous areas contains 16 to 127  $\gamma$  of selenium per 100 cc. Moxon (3) observed that selenium was carried in the milk of selenized animals to such an extent that nursing calves often showed all the prominent symptoms of "alkali" disease.

Organoselenium as obtained from seleniferous grains (Franke and Potter (4)) and inorganic selenium, when administered to experimental animals, have been shown to pass into the proteins of liver<sup>1</sup> (Westfall and Smith (5)), muscle,<sup>1</sup> the plasma fractions<sup>1</sup> (Westfall and Smith (5)), and hemoglobin.<sup>1</sup> In view of the fact that there is a certain fixation of selenium in tissue proteins, the question may be raised whether selenium of the milk exists in the milk proteins and, if so, in what protein fractions. In order to obtain information about the nature of selenium in the milk of selenium-injected white rats, various experiments were carried out.

The experiments reported here were carried out with the radioactive isotope of selenium. With the use of radioselenium which has the advantages of a half life of 50 days with a specific  $\gamma$ -ray emission of 0.05 and 0.21 m.e.v., microdeterminations of selenium were made with far greater accuracy than are possible by any known chemical analytical procedure. The extreme sensitivity of the method made possible quantitative detections of traces of selenium.

## EXPERIMENTAL

It was first established, as it has been in domestic animals, that selenium passes through the mammary glands of the white rat. This was accom-

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<sup>1</sup> Unpublished data of the author.

plished in the following manner. Five lactating rats, having three to six pups, 2 to 3 days old, were injected either with single or multiple, subcutaneous subtoxic amounts of sodium selenate containing radioselenium. 24 hours after the last parental injection, the pups were sacrificed by decapitation and wet ashed separately, radioactivity of the ash was determined on a dipping Geiger-Muller counter of a scale-of-four.

Studies on the distribution of selenium in the milk necessitated the fractionation of the various milk proteins. The milk for these distribution studies was collected as follows. Two lactating white rats having six and nine pups respectively, 10 to 14 days of age, were injected with sodium selenate containing radioselenium. The pups were separated from their mothers for 12 to 18 hours, after which they were returned and allowed to suckle for 1 to 3 hours. The pups were then sacrificed, the stomachs excised, and the stomach contents, which consisted of milk curd, removed. The milk of the two litters was pooled separately and the milk samples were agitated with a small amount of benzoic acid to break up the curd (Mayer (6)).

Total proteins of the milk obtained from Litter 1 were precipitated with trichloroacetic acid. The milk from Litter 2 was divided into three aliquots and three protein fractions were obtained with the method of Moir (7). (a) *Casein* was obtained by isoelectric precipitation at pH 4.6, (b) *casein and globulin* fractionation was obtained by saturating the milk solution with magnesium sulfate at 20°, and (c) *total protein* was precipitated by heating with 4 per cent trichloroacetic acid.

On each of the above protein fractionations radioactive determinations were made on the filtrate and precipitate. All the precipitates were washed twice.

The globulin content of the milk was obtained by subtracting the casein fraction from the casein plus globulin fraction. Albumin was determined by the analysis of the filtrate from the casein plus globulin precipitation. In this manner it was possible to estimate the distribution of selenium in total proteins, albumin, globulin, and casein fractions.

#### RESULTS AND DISCUSSION

The results from these two series of experiments will be considered under separate headings, *i.e.*, evidence that selenium passes through the mammary glands of the white rat, and distribution of selenium in the various milk proteins.

*Presence of Selenium in Milk of White Rat*—The results for this section are shown in Table I, where the concentration of radioactive selenium in the pups is expressed both as per cent dose per pup and per cent dose per litter. Upon inspection of Table I it will be noted that after both single and multiple subcutaneous injections of sodium selenate 0.33 to 2.3 per

cent of the dose per pup or 2.0 to 9.3 per cent of dose per litter was transferred through the milk of the lactating mother to the suckling young. It must be borne in mind that the actual amount of selenium transferred through the mammary glands of the mother to the young during the experimental period outlined here is not truly represented in the values expressed as per cent dose per litter. These values represent a fraction of the actual amount of selenium secreted, the exact amount transferred through the glands is undoubtedly greater because part of the selenium received by the young via milk is excreted through the kidney, gastrointestinal tract, and lungs. The values expressed as per cent of the dose per litter are representative of the amount of selenium in the litters 24 hours after the last parental injection. These data, however, are sufficient to show that selenium, when administered to lactating mothers as an inorganic salt, is transferred through the mammary glands of the white rat and that at least 2.5

TABLE I

*Passage of Selenium (Radio) through Mammary Glands of White Rat after Subcutaneous Injection of Sodium Selenate ( $\text{Na}_2\text{SeO}_4$ )*

Experiment	No. of parental injections	Total selenium injected	Total count injected	No. of pups suckled	Per cent dose administered per pup		Per cent dose administered per litter
					Average	Range	
A	4	1.12	324	4	2.30	1.8-3.4	9.3
B	3	0.62	544	3	1.60	1.4-2.0	5.0
C	3	0.76	3960	6	0.42	0.36-0.50	2.5
D	1	0.58	1555	6	1.61	0.36-0.71	3.7
E	1	0.58	1555	6	0.33	0.20-0.43	2.0

to 9.3 per cent of the dose is present in the suckling young 24 hours after the last parental injection.

The list of animals that may secrete selenium through the mammary glands is, therefore, extended to include the white rat.

The mammary gland of one lactating animal was found to contain 0.058 per cent of the dose per gm. of tissue 24 hours after the last injection. This value corresponds to about the value found for per cent of the dose per gm. of pup and about a fourth the value for liver (0.208) in the same time interval.

*Distribution of Selenium in Various Milk Proteins*—The results obtained for this section are given in Table II. Perhaps the most outstanding finding here is that 91 per cent of the selenium present in the milk obtained from Rat 1 was in the protein fraction, while in Rat 2 all the selenium was present in protein fractions.

Of importance here is the fact that *inorganic selenium* is converted to



some *organoselenium protein complex*. This conversion of inorganic selenium to organoselenium has been shown to occur in other tissue proteins, *z e*, the liver, muscle, hemoglobin, and plasma fractions. The value and significance of these findings in relationship to the metabolism of selenium and the closely related sister substance, sulfur, cannot be stated but must await further elucidation and experimentation. Smythe and Halliday (8) were able to demonstrate in *in vitro* studies that inorganic sulfur (radio-sulfur) as sodium sulfide may be converted in the presence of a suitable liver enzyme to cysteine sulfur. This established without a doubt that inorganic sulfur was converted to organic sulfur. Recently, Dziewiatkowski (9) has demonstrated that sulfide sulfur containing radioactive sulfur ( $S^{35}$ )

TABLE II

*Presence of Selenium (Radio) in Rat Milk Proteins after Subcutaneous Injections of Sodium Selenate ( $Na_2SeO_4$ )*

	Rat 1	Rat 2
Volume of milk, cc	0.5	2.0
Counts per cc	26.0	46.1
Dose per cc, %	0.515	0.913
Counts in protein fraction	11.3	64.8
"    " non-protein fraction	1.0	0.0
Counts recovered, %	94.5	72.0
"    in casein, %		53.8 (Corrected)
"    " albumin and globulin, %		46.4
"    " casein " " %		60.8
"    " albumin, %		39.5
"    " globulin, %		6.9

Rats 1 and 2 were injected with 5050 counts of radioseelenium as sodium selenate

was utilized by the intact rat for the synthesis of cystine. The radioactive sulfur from sulfide was found to be present in the cystine isolated from hair, liver, skeletal muscle, and skin.

It may be that a similar mechanism may take place in the experiments described here in the conversion of inorganic selenium to organoselenium.

The protein fractionation experiments carried out on the milk obtained from Rat 2 show that approximately 54 per cent of the selenium present in the milk is in the casein fraction, while smaller amounts, namely 40 and 7 per cent, are in the albumin and globulin fractions, respectively. It is of interest here to point out that the presence of selenium in milk protein is not confined to any one particular protein fraction but may be detected in all the fractions, as was observed in the plasma experiments<sup>1</sup>. The distribution of selenium in the various protein fractions of milk is more or

less proportional to the size of the fraction. Casein, which is the largest protein fraction of milk (Cox and Mueller (10)) concentrates the most selenium, while lesser amounts of selenium are present in the smaller milk protein fractions, albumin and globulin.

#### SUMMARY

It has been demonstrated that after subtoxic subcutaneous injections of selenium (radioactive) as sodium selenate, selenium passes through the mammary gland into the milk of the white rat. Further, it has been found that administered inorganic selenium was converted into organoselenium in the protein fraction of milk because approximately 54 per cent of the selenium present in the milk was in the casein fraction, while smaller amounts, namely 40 and 7 per cent, were in the albumin and globulin fractions, respectively.

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# THE METABOLIC INTERRELATIONSHIP BETWEEN TRYPTOPHAN, PYRIDOXINE, AND NICOTINIC ACID, FORCED FEEDING STUDIES IN RATS\*

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## PLATE 1

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The addition of corn products to a synthetic low protein diet very low in nicotinic acid has been reported by Krehl and coworkers (1, 2) to depress growth in rats. This growth retardation could be prevented by tryptophan as well as by nicotinic acid. These findings have been confirmed in this laboratory by the paired feeding technique (3) and by other investigators (4, 5). The interchangeability of nicotinic acid and tryptophan is not peculiar to corn-supplemented rations, since a similar relationship has been demonstrated with non-corn rations which are low in both tryptophan and nicotinic acid (6, 7). The Wisconsin group has indicated that intestinal microorganisms may play a significant part in the deficiency syndrome, since the growth-depressing effect of corn can be greatly modified by the use of carbohydrates previously shown to favor intestinal synthesis (7).

An increased excretion of nicotinic acid and its derivatives has been shown to result from the administration of tryptophan (4, 5, 8, 9), which has been interpreted to indicate that tryptophan may function as a metabolic precursor of nicotinic acid. The studies which have been reported on the increased excretion of nicotinic acid following tryptophan feeding have been made with adult rats on basal diets which contained tryptophan. It was thought desirable to compare the excretion of nicotinic acid of growing rats on an acid-hydrolyzed casein diet containing a minimum of tryptophan (0.24 mg per gm of diet) with that obtained with an adequate amount of tryptophan (20 mg per rat per day) and to determine whether there is any relationship between the excretion of nicotinic acid and the gain in weight.

It has been shown that in pyridoxine-deficient rats tryptophan is not normally metabolized, resulting in the excretion of xanthurenic acid in the urine (10-13). It was of interest, therefore, to study the effect of a pyridoxine deficiency on the excretion of nicotinic acid and its methylated

\*This material was presented at the 112th meeting of the American Chemical Society at New York, September, 1947.

derivative, N<sup>1</sup>-methylnicotinamide, associated with the feeding of tryptophan<sup>1</sup>

In the investigation reported here, the metabolic interrelationship between tryptophan, pyridoxine, and nicotinic acid was studied with an acid-hydrolyzed casein diet deficient in tryptophan as well as pyridoxine and nicotinic acid

#### EXPERIMENTAL

*Basal Ration*—The composition of the basal ration is shown in Table I "Vitamin-free" casein (Labco) was extracted three times with hot 95 per cent ethanol and then completely hydrolyzed<sup>2</sup> with sulfuric acid by the method of Beig and Rose (16) The hydrolysate was first put through a spray drier<sup>3</sup> which reduced the moisture content to about 5 per cent, and then was completely dried in a vacuum oven at about 70° 2 per cent of unhydrolyzed "vitamin-free" casein was added to supply streptogenin (17), which is destroyed by acid hydrolysis All the vitamins, with the exception of nicotinic acid and pyridoxine, were provided in excess of the requirement The very small amounts of tryptophan and nicotinic acid supplied by the basal ration are shown in Table I The amount of pyridoxine present in the basal ration was less than could be measured accurately

*Feeding Procedure*—Originally, weanling rats (Sprague-Dawley strain) were fed variable amounts of ground Purina dog checkers until the weight of each animal was within 2 gm of 60 gm They were then separated into ten groups of six each, with uniform distribution as to sex and litter Thus, eight groups provided all possible combinations of the three supplements tryptophan, nicotinic acid, and pyridoxine In addition, there were two groups receiving 2 per cent succinylsulfathiazole, tryptophan, and pyridoxine, one group without and the other with nicotinic acid

All the animals, regardless of the supplement, ate only 3 to 5 gm of the diet and consequently lost weight The addition of large amounts of thiamine and of sodium glutamate did not increase the food consumption After 2 weeks a regimen of forced feeding was started Most of the rats receiving no tryptophan died the 1st day of forced feeding, and the remainder died within a couple of days The animals receiving tryptophan survived 1 week of forced feeding, but they were so emaciated from the

<sup>1</sup> After this study was started two laboratories published results of experiments on the effect of vitamin B<sub>6</sub> deficiency on the conversion of tryptophan to nicotinic acid and N<sup>1</sup>-methylnicotinamide (14, 15) Again, however, the basal rations contained an adequate amount of tryptophan for growth

<sup>2</sup> Grateful acknowledgment is made to Dr M Womack for aid in the preparation of the acid-hydrolyzed casein, and to Dr W C Rose for the use of equipment in the Department of Chemistry

<sup>3</sup> This was done in the Division of Dairy Manufactures by Mr V L Swearingen

first 2 weeks of poor food consumption that it was deemed advisable to start an entire series of new animals

The weights of 60 young rats<sup>4</sup> were adjusted to within 2 gm of 80 gm by feeding variable amounts of ground Purina dog checkers. The rats were then separated into ten groups of six each as in the first experiment

TABLE I  
*Composition of Basal Ration*

	<i>per cent</i>
Acid hydrolyzed casein (Labco, extracted 3 times with 95% ethanol)	20 0
Labco casein	2 0
Salts 446 (Illinois)*	5 0
Corn oil	3 0
DL-Methionine	0 6
Sucrose	69 4
2 % sulfasuxidine added at expense of sucrose	
<b>Level of incorporated vitamins</b>	
	<i>mg per 100 gm</i>
Thiamine	0 25
Riboflavin	0 50
Calcium pantothenate	2 0
Choline chloride	100 0
Inositol	10 0
p Aminobenzoic acid	50 0
Biotin	0 01
Folic acid	0 025
2-Methylnaphthoquinone	0 1
Halibut liver oil (diluted 1 : 2 with corn oil) at level of 2 drops per wk, with $\alpha$ -tocopherol included at 0.5 mg per drop	
<b>Basal ration supplied per gm</b>	
Tryptophan	0.24 mg
Nicotinic acid	0.042 $\gamma$

\* The composition of Salts 446, in gm, is as follows: NaCl 243.198,  $K_2C_2H_3O_7 \cdot H_2O$  533.0,  $K_2HPO_4$  174.0,  $CaHPO_4 \cdot 2H_2O$  800.0,  $CaCO_3$  368.0,  $MgCO_3$  92.0,  $FeC_2H_3O_7 \cdot 3H_2O$  36.0,  $CuSO_4 \cdot 5H_2O$  0.4,  $MnSO_4$  2.8,  $K_2Al(SO_4)_3 \cdot 24H_2O$  0.2, KI 0.1,  $CoCl_2 \cdot 6H_2O$  0.2,  $ZnCO_3$  0.1, NaF 0.002, total 2250.0

The basal ration was well mixed with an equal amount of distilled water. The desired amount of this slurry was taken up into a graduated 10 cc hypodermic syringe to which was attached an ordinary hypodermic needle, the tip of which was blunted with a file and coated with a protective knob

<sup>4</sup> Obtained from the Department of Chemistry

of solder (18) After safely inserting this needle into the rat's stomach, the measured amount of diet was gently injected As a check on the actual amount fed, the rats were weighed immediately before and after feeding The rats were fed three times daily, at about 5 hour intervals, with the supplement included in the first feeding The amount of basal ration, supplement, and the average weekly gain in weight of each group are shown in Table II

TABLE II  
*Average Weekly Gain in Weight in Gm (with Standard Errors)*

Wk	Tryptophan				No tryptophan				2 per cent sulfasuxidine	
	Nicotinic acid		No nicotinic acid		Nicotinic acid		No nicotinic acid		Tryptophan + pyridoxine	
	Pyri doxine	No pyri doxine	Pyri doxine	No pyri doxine	Pyri doxine	No pyri doxine	Pyri doxine	No pyri doxine	No nic otinic acid	Nic otinic acid
1st	11.8 ±1.4	10.8 ±1.3	9.9 ±1.0	10.1 ±1.2	10.7 ±2.0	8.6 ±0.4	10.6 ±1.0	9.6 ±2.2	14.1 ±1.0	*15.9 ±1.0
2nd	15.4 ±1.7	15.7 ±1.0	13.9 ±1.2	15.4 ±1.5					16.5 ±1.9	15.9 ±1.3
3rd	16.8 ±1.1	16.3 ±2.1	17.2 ±1.0	17.8 ±0.9					17.7 ±2.1	18.7 ±1.2
Total	43.5 ±3.7	43.3 ±4.2	41.2 ±2.9	43.6 ±3.1					48.1 ±3.4	†50.5 ±1.9

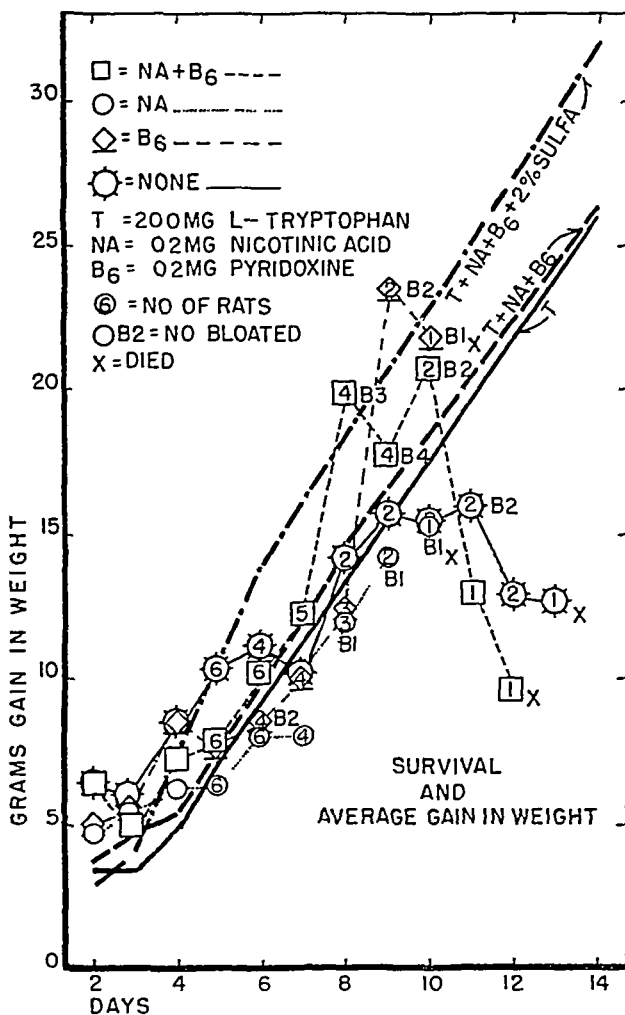
Amount of ration given		Daily supplement		Probability of chance outcome
wk	gm		mg	
1st, 2nd	7.5	L-Tryptophan	20.0	* <0.001
3rd	9.75	Nicotinic acid	0.2	† 0.0077
		Pyridoxine	0.2	

*Gain in Weight*—Statistical examination<sup>5</sup> of the data by the method of analysis of variance (19) indicated no significant effect of any of the supplements upon gain in weight. However, porphyrim-caked whiskers and acrodynia of the mouth, paws, and tail were evident in the pyridoxine-deficient rats on the 13th day and all of the tryptophan-deficient rats were dead by this time.

The presence of 2 per cent succinylsulfathiazole in the diet resulted in a

<sup>5</sup> Grateful acknowledgment is made to Dr. K. M. Blaxter, Commonwealth Fellow on leave of absence from the Ministry of Agriculture and Fisheries, Great Britain, for advice and instruction in the statistical treatment of the data.

significantly greater gain in body weight during the 1st week, but not in subsequent weeks. Thus, there was a greater slope (Text-fig 1) for the growth curve of the succinylsulfathiazole-fed rats during the 1st week, but



TEXT-FIG 1

thereafter the slope was about the same as for the other groups. Elvehjem and his group at Wisconsin (20) have observed a similar beneficial effect of sulfasuxidine on the weight of chicks when folic acid was included in the diet.



*Tryptophan Deficiency*—The tryptophan-deficient animals assumed a hunch-backed position and were unthrifty looking, with the mouth, paws,

TABLE III—Average Urinary

The results are expressed as micrograms per rat per day

		Tryptophan					
		Nicotinic acid				No nicotinic acid	
		Pyridoxine		No pyridoxine		Pyridoxine	No pyridoxine
1st wk	Tryptophan	152 0 ± 38 2		126 6 ± 50 3		244 6 ± 36 1	159 8
	Excretion, %	0 70		0 58		1 12	0 7
	Nicotinic acid	23 0 ± 1 8		20 8 ± 1 4		14 4 ± 0 2	13 5
	Conversion or recovery, %	4 30		3 65		0 066	0 0
	N <sup>1</sup> -Methylnicotinamide	139 5 ± 18 9		76 5 ± 17 8		29 1 ± 0 6	20 0
	Conversion or recovery, %	55 20		25 75		0 133	0 1
2nd wk	Xanthurenic acid	0		220 0 ± 111 4		0	0
	Conversion, %	0		1 01		0	0
	Tryptophan	163 8 ± 28 5		158 8 ± 43 2		189 4 ± 46 6	120 4
	Excretion, %	0 73		0 71		0 85	0 5
	Nicotinic acid	24 8 ± 3 4		22 9 ± 1 1		14 9 ± 0 4	14 7
	Conversion or recovery, %	4 95		4 10		0 067	0 0
3rd wk *	N <sup>1</sup> -Methylnicotinamide	140 2 ± 19 1		64 7 ± 11 3		27 2 ± 1 3	31 3
	Conversion or recovery, %	56 5		16 7		0 122	0 1
	Xanthurenic acid	0		420 0 ± 177 2		0	216 6
	Conversion, %	0		1 88		0	0 9
	Tryptophan	2760 0 ± 823 8		434 5 ± 34 5		1285 6 ± 277 8	2090 0
	Excretion, %	2 70		0 42		1 26	2 0
3rd wk *	Nicotinic acid	39 4 ± 5 4		26 7 ± 5 4		22 4 ± 0 6	19 9
	Conversion or recovery, %	8 50		3 40		0 022	0 0
	N <sup>1</sup> -Methylnicotinamide	525 6 ± 78 4		443 5 ± 125 5		129 6 ± 14 6	78 1
	Conversion or recovery, %	198 00		182 35		0 127	0 1
	Xanthurenic acid	0		6083 5 ± 1416 5		0	2833
	Conversion, %	0		5 95		0	2

\* Urine was collected for 24 hours following a dose of 100 mg of L-tryptophan

and abdominal area denuded. They progressively manifested the following symptoms: bloat, diarrhea, convulsions with screeching, and finally death. After forced feeding all tryptophan-deficient animals displayed the following peculiar behavior: (1) pawing the face as though trying to with-

draw the food, (2) crawling around the edge of the cage with the side of the face resting on the floor of the cage, (3) rearing up on the hind legs

etion (with Standard Errors)

Donne	No tryptophan				2 per cent sulfasuxidine			
	Nicotinic acid		No nicotinic acid		Tryptophan + pyridoxine			
	Pyridoxine	No pyridoxine	Pyridoxine	No pyridoxine	No nicotinic acid		Nicotinic acid	
± 31 1	39 4 ± 22 1	18 5 ± 9 5	22 0 ± 2 1	33 5 ± 9 5	187 6 ± 48 9		140 0 ± 29 5	
	2 19	1 03	1 22	1 86	0 86		0 64	
± 0 9	13 8 ± 3 2	10 6 ± 0 1	7 2 ± 3 0	7 8 ± 1 2	13 8 ± 1 2		16 2 ± 0 7	
	3 30	1 40	0 400	0 433	0 063		1 20	
± 5 2	43 0 ± 26 6	28 0 ± 8 0	22 0 ± 3 1	16 8 ± 1 8	26 2 ± 2 6		121 2 ± 13 8	
	10 50	5 60	1 222	0 933	0 120		47 50	
	0	0	0	0	0		0	
	0	0	0	0	0		0	
± 36 3					136 2 ± 15 9		125 2 ± 12 0	
					0 61		0 56	
± 0 4					16 4 ± 0 7		16 0 ± 2 0	
					0 074		0	
± 6 5					32 0 ± 2 4		123 6 ± 6 3	
					0 143		45 80	
± 164 1					0		0	
					0		0	
± 932 3					2044 6 ± 894 4		2187 0 ± 1047 1	
					2 0		2 14	
± 2 1					27 0 ± 2 3		53 1 ± 6 1	
					0 026		13 05	
± 30 0					191 2 ± 42 2		553 2 ± 71 7	
					0 187		181 00	
± 793 5					0		0	
					0		0	

and pawing frantically with the front paws, (4) after 5 to 10 minutes they would go to sleep, with a tendency to sleep on the side

The extent of bloat may be seen in the group receiving pyridoxine, which showed an average gain of 11 gm per rat from the 8th to the 9th day. The

first fatality occurred on the 6th day, and on the 7th day there was at least one dead in all four groups receiving no tryptophan. All the animals from the two groups receiving either nicotinic acid or pyridoxine were dead on the 10th day. The animal which lived the longest, 13 days, was from the group receiving the basal ration without any supplement.

In a supplementary experiment to determine whether tryptophan could cure as well as prevent the syndrome, twelve young rats (weighing 75 to 88 gm) were forcibly fed the tryptophan-deficient basal ration plus nicotinic acid and pyridoxine. One animal died on the 2nd day and five more had died by the morning of the 3rd day. The animals had an unkempt appearance, bloat, diarrhea, and fits. A supplement of 40 mg of tryptophan was then given to the remaining animals; one died the same day, but in the others the daily supplement of tryptophan eliminated the deficiency symptoms.

On postmortem examination the stomach and intestines of the tryptophan-deficient animals were found to be greatly distended and filled with liquid and gas. The liver was light colored. One rat from the group receiving nicotinic acid and pyridoxine was sacrificed for histological study on the 10th day of experiment.<sup>6</sup>

The cells (Fig. 1) show fatty degeneration in the cytoplasm. They are swollen, rounded, and highly vacuolated, resulting in a tenuous cytoplasm resembling chicken-coop wire. Another interesting feature is the appearance of the nuclei, which contain large masses of chromatin and have an enlarged nucleolus.<sup>7</sup>

*Excretion Studies, Methods*—Individual urinary collections were made for 24 hours in metabolism cages during the 2nd and 3rd weeks and after an oral dose of 100 mg of L-tryptophan during the 4th week. To neutralize a possible effect of time, urine was collected from an equal number of rats from each group on any particular day. The bladder was emptied at the beginning and end of the collection period by applying pressure. The urine was analyzed for apparent free tryptophan (21) and for nicotinic acid (22), with *Lactobacillus arabinosus* as the test organism. The N<sup>1</sup>-methyl-nicotinamide was determined by the acetone-fluorimetric method of Huff and Perlzweig (23), and xanthurenic acid<sup>8</sup> by the colorimetric method of Rosen *et al* (14).<sup>9</sup>

<sup>6</sup> Grateful acknowledgment is made to Dr. A. V. Nalbandov, Dr. F. B. Adamstone, and Dr. C. C. Morrill for the preparation and interpretation of the liver sections.

<sup>7</sup> A more complete study of the histologic changes in tryptophan deficiency produced by forced feeding is planned.

<sup>8</sup> The xanthurenic acid was kindly furnished by Dr. J. W. Huff of Sharp and Dohme, Inc.

<sup>9</sup> I wish to thank Lucille D. van Ghyl, Barbara Chase, and Shirley Spaeth for assistance in some of these determinations.

*Results*—Average urinary excretions, expressed as micrograms per rat per day, are shown in Table III. The values given are the group means with their standard errors. The amount of nicotinic acid excreted when no nicotinic acid was added was calculated in terms of a percentage conversion from tryptophan on a weight basis. This amount was then subtracted from the nicotinic acid excretion for the corresponding group receiving nicotinic acid, and the per cent recovery of added nicotinic acid was calculated. The same calculations were made for N<sup>1</sup>-methylnicotinamide. In Table IV the effect of each supplement, both in the presence and absence of the other supplements, is computed, and the difference is expressed as a percentage of the amount excreted when the particular supplement was not given. The mean differences were analyzed statistically by the *t* test (19).

*Effect of Tryptophan*—It is interesting that the addition of tryptophan resulted in the greatest increase in tryptophan excretion when nicotinic acid was not added. Although the general result of adding nicotinic acid was a lesser excretion of tryptophan, the differences are not statistically significant, and there are even some cases of an increased excretion.

The addition of 20 mg of L-tryptophan increased the excretion of nicotinic acid from 78 to 86 per cent, regardless of the presence or absence of nicotinic acid or pyridoxine. The increase in N<sup>1</sup>-methylnicotinamide due to the addition of tryptophan was considerably greater when nicotinic acid was present (204 per cent) than when it was lacking (39 per cent).

*Effect of Pyridoxine*—There was no significant effect of pyridoxine on tryptophan excretion in the absence of added tryptophan and at the 20 mg level, although the trend was toward a higher excretion. When 100 mg of L-tryptophan were fed, however, pyridoxine greatly increased the excretion of tryptophan in the presence of nicotinic acid, but when nicotinic acid was lacking, pyridoxine decreased the excretion of tryptophan. The latter was not statistically significant ( $P = 0.202$ ).

Pyridoxine had no significant effect either on the percentage conversion of tryptophan to nicotinic acid or of tryptophan to N<sup>1</sup>-methylnicotinamide. However, it is significant that there was a greater excretion of added nicotinic acid in the form of N<sup>1</sup>-methylnicotinamide when pyridoxine was present. When 100 mg of L-tryptophan were given, however, this difference was not statistically significant ( $P = 0.300$ ), probably because of the variability in excretion of N<sup>1</sup>-methylnicotinamide that was obtained.

*Effect of Nicotinic Acid*—It is of great significance that when 100 mg of L-tryptophan were fed, 181 to 198 per cent of added nicotinic acid was recovered as N<sup>1</sup>-methylnicotinamide, even after the amount synthesized and excreted in the absence of nicotinic acid was subtracted. These figures indicate that in the presence of nicotinic acid there was actually a greater conversion of tryptophan to N<sup>1</sup>-methylnicotinamide than when nicotinic

acid was lacking. The increase in N<sup>1</sup>-methylnicotinamide due to the addition of nicotinic acid was greatest in the presence of tryptophan and least in the absence of tryptophan. At the 20 mg level of tryptophan this increase was also greater in the presence of pyridoxine than in its absence, but this was not so at the 100 mg level of L-tryptophan.

TABLE IV—Isolation of

			Effect of tryptophan				
			Nicotinic acid		Pyridoxine		Nicot
			+	-	+	-	+
2nd wk	Tryptophan	Difference, %	381.2	628.6	545.9	150.8	31.9
		P*	0.001	<0.0005	<0.0005	0.0028	0.25
	Nicotinic acid	Difference, %	79.5	86.0	78.1	86.4	17.2
		P	<0.0005	0.0023	0.0033	<0.0005	0.097
	N <sup>1</sup> -Methylnicotinamide	Difference, %	201.2	39.4	159.4	126.6	74.6
		P	0.0023	0.018	0.0016	0.01	0.031
	Xanthurenic acid	Difference, %	220	0	0	220	-220
		P	0.011			0.041	0.041
3rd wk	Tryptophan	Difference, %					3.1
		P					>0.45
	Nicotinic acid	Difference, %					8.3
		P					0.30
	N <sup>1</sup> Methylnicotinamide	Difference, %					116.7
		P					<0.00
	Xanthurenic acid	Difference, %					-420
		P					<0.00
After 3rd wk †	Tryptophan	Difference, %					535.2
		P					0.02
	Nicotinic acid	Difference, %					47.6
		P					0.08
	N <sup>1</sup> Methylnicotinamide	Difference, %					18.5
		P					0.30
	Xanthurenic acid	Difference, %					-6083.5
		P					<0.00

\* P = probability of chance outcome

† Urine was collected for 24 hours following a dose of 100 mg of L-tryptophan

The presence of nicotinic acid also resulted in a greater conversion of tryptophan to xanthurenic acid in pyridoxine deficiency, and during the 2nd week there was no detectable excretion of xanthurenic acid in the group which did not receive nicotinic acid. During the 3rd week both groups converted more tryptophan to xanthurenic acid, but there was almost twice as much xanthurenic acid excreted, as well as a greater amount of tryptophan, by the group receiving nicotinic acid.

*Effect of Succinylsulfathiazole*—Succinylsulfathiazole had no significant effect on the excretion of tryptophan. It had a variable effect, which is not statistically significant, on the percentage conversion of tryptophan to nicotinic acid, but the recovery of added nicotinic acid was 29.6 and 35.5 per cent less, and during the 3rd week there was a striking failure to re-

*Is on Urinary Excretion*

of pyridoxine			Effect of nicotinic acid						Effect of sulfasuxidine	
Tryptophan			Pyridoxine		Tryptophan		Sulfasuxidine		Nicotinic acid	
-	+	-	+	-	+	-	+	-	+	-
37.9	38.5	18.1	-28.2	-24.9	-31.1	4.3	-25.4	-37.9	-7.9	-23.3
0.083	0.094	0.365	0.106	0.225	0.069	>0.45	0.216	0.061	0.405	0.188
1.4	9.6	14.1	70.3	47.4	57.0	62.7	17.4	59.7	-29.6	-4.2
>0.45	0.097	0.294	0.0042	<0.0005	<0.0005	0.04	0.061	0.0013	0.0045	0.312
22.2	66.1	45.1	257.1	150.0	299.3	83.0	362.6	379.4	-13.1	-10.0
0.086	0.012	0.249	0.0016	0.006	<0.0005	0.147	<0.0005	<0.0005	0.230	0.154
0	-220	0	0	220	220	0	0	0	0	0
	0.041			0.041	0.041					
57.3			-13.5	31.9			-8.1	-13.5	-23.6	-28.1
0.139			0.326	0.258			0.299	0.326	0.125	0.156
1.4			66.4	55.8			-2.4	66.4	-35.5	10.1
0.375			0.0097	<0.0005			0.427	0.0097	0.028	0.059
13.1			415.4	106.7			286.3	415.4	-11.8	17.6
0.277			<0.0005	0.019			<0.0005	<0.0005	0.219	0.061
16.6			0	93.9			0	0	0	0
<0.0005				0.214						
38.5			114.7	-79.2			7.0	114.7	-20.8	59.0
0.202			0.067	0.073			>0.45	0.067	0.339	0.202
12.6			75.9	34.2			96.7	75.9	34.8	20.5
0.144			0.007	0.146			0.0033	0.007	0.069	0.047
64.5			305.6	462.8			189.3	305.6	5.3	47.5
0.087			<0.0005	0.02			0.0023	0.0007	0.400	0.103
33.3			0	114.7			0	0	0	0
<0.0005				0.047						

cover any nicotinic acid as such. When 100 mg of L-tryptophan were fed, succinylsulfathiazole surprisingly gave a greater conversion of tryptophan to nicotinic acid and also a greater recovery of added nicotinic acid, although the latter is not statistically significant ( $P = 0.069$ ). Succinylsulfathiazole had no depressing effect, either on the conversion of tryptophan to N<sup>1</sup>-methylnicotinamide or the recovery of added nicotinic acid in the form of N<sup>1</sup>-methylnicotinamide, in fact, when 100 mg of L-tryptophan

were fed, the excretion values were higher, but the differences were not statistically significant

*Effect of Time*—The only fundamental differences in the urinary excretion between the 2nd and 3rd weeks, *i.e.* the effect of time, are an increase in the formation of xanthurenic acid and an increase in the conversion of tryptophan to nicotinic acid in the presence of succinylsulfathiazole, which is on the border line of statistical significance ( $P = 0.059$ )

*Comparison of Tryptophan Levels*—It is worth while to compare the results obtained with an adequate amount of tryptophan (20 mg) with those of an excess of tryptophan (100 mg). At the higher level of tryptophan there was a greater percentage of tryptophan excreted except in the pyridoxine-deficient group receiving nicotinic acid, in which there was a very large per cent of tryptophan excreted as xanthurenic acid, and, as at the lower level, this xanthurenic acid excretion was greater than the combined excretion of tryptophan and xanthurenic acid when nicotinic acid was not added. In both pyridoxine-deficient groups there was a greater per cent of tryptophan converted to xanthurenic acid at the 100 mg level than at the 20 mg level.

As the amount of tryptophan was increased from 1.8 to 20 mg and 100 mg, there was an increase in the actual amount of nicotinic acid excreted, but the percentage conversion was progressively less. The same was true for  $N^1$ -methylnicotinamide with the exception that when pyridoxine was present the per cent conversion of tryptophan to  $N^1$ -methylnicotinamide was the same at both levels of added tryptophan. The recovery of added nicotinic acid in the form of  $N^1$ -methylnicotinamide ranged from only 16.7 to 56.5 per cent at the 20 mg level of tryptophan, while at the 100 mg level of tryptophan the recovery was in considerable excess of the amount added, with the range 181.0 to 198.0 per cent.

#### DISCUSSION

The apparent interchangeability of nicotinic acid and tryptophan in promoting growth has been demonstrated (1-7) only with diets containing marginal amounts of tryptophan. However, the present study shows that, while tryptophan can entirely replace nicotinic acid, nicotinic acid cannot replace tryptophan, which is one of the ten amino acids essential for the growth and maintenance of rats. Thus, in the absence of added tryptophan (only 1.8 mg per day) nicotinic acid had no effect on the survival time.

The failure to obtain a beneficial effect of pyridoxine on gain in weight is surprising, especially since forced feeding should have exaggerated the deficiency of pyridoxine. The answer does not seem to lie in the duration of the experiment, for definite gross symptoms of pyridoxine deficiency were obtained in all animals not receiving this vitamin, and the excretion

of xanthurenic acid confirmed the existence of the metabolic defect associated with pyridoxine deficiency. The seeming ability of the tryptophan-deficient animals to show a gain in weight for the 1st week which was not significantly less than that of the tryptophan-supplemented animals may have been due to complication with bloat.

In a previous publication from this laboratory (3) it was postulated that the interrelationship between tryptophan and nicotinic acid when the dietary intake of both is low may be analogous to the metabolic interrelationship of methionine and choline. Singal and coworkers (9) suggested a similar analogy, the methionine-cystine relationship, to describe the interchangeable rôle of tryptophan and nicotinic acid. The large increase in the urinary excretion of nicotinic acid and its derivatives following the addition of tryptophan to various diets in the albino rat (4, 5, 8, 9), cotton-rat and horse (24), pig (25), man (26, 27), and dog (28) has been interpreted by these investigators to indicate that tryptophan is the dietary precursor of niacin. Although the evidence is circumstantial, the present study supports this conclusion as the most probable explanation of the increased urinary excretion of niacin following tryptophan administration.

The following facts, that (1) tryptophan supplementation resulted in increased urinary excretion of nicotinic acid and  $N^1$ -methylnicotinamide on a diet extremely low in nicotinic acid, (2) this excretion continued for over 3 weeks, (3) there was an increase in nicotinic acid and  $N^1$ -methylnicotinamide excretion when tryptophan was increased, (4) over 200 per cent of added nicotinic acid was recovered when 100 mg of L-tryptophan were given, indicate that tryptophan is being converted into nicotinic acid and  $N^1$ -methylnicotinamide rather than that tryptophan is sparing or mobilizing the body's store of this vitamin. To provide direct proof for the probable conversion of tryptophan to niacin a tracer experiment, with tryptophan labeled with isotopic carbon or nitrogen, has been suggested (29, 30).

Ellinger and Benesch (31) have reported that succinylsulfathiazole diminished the urinary output of  $N^1$ -methylnicotinamide by an average of 60 per cent in man and interpreted this as demonstrating a biosynthesis of nicotinamide in the gut. Later (32) Ellinger reported that this drug also reduced the excretion of  $N^1$ -methylnicotinamide in the rat. Nayyar *et al* (33) found no reduction in the excretion of  $N^1$ -methylnicotinamide upon the administration of succinylsulfathiazole, and suggested that the difference may have been due to the nature of the basal diets used. In this study the results with succinylsulfathiazole indicate that the conversion of tryptophan to nicotinic acid and  $N^1$ -methylnicotinamide is probably not dependent upon the synthetic activities of intestinal microorganisms.

The only significant effect of pyridoxine on niacin excretion observed in this study was an increase in  $N^1$ -methylnicotinamide when nicotinic acid



and 20 mg of tryptophan were also added, but not when nicotinic acid was absent, and at the 100 mg level of tryptophan there was no effect of pyridoxine even when nicotinic acid was present. These observations suggest that the synthesis of N<sup>1</sup>-methylnicotinamide from tryptophan was increased by the addition of nicotinic acid, and this increase was greatest in the presence of pyridoxine at the 20 mg level of tryptophan, but any beneficial effect of pyridoxine is probably dependent upon the presence of an adequate amount of nicotinic acid.

Rosen, Huff, and Peilzweig (14) reported that pyridoxine deficiency affects adversely the transformation of tryptophan to nicotinic acid in the rat. As the pyridoxine deficiency became more acute, as measured by the increase in xanthurenic acid, they found a progressive decrease in the excretion of N<sup>1</sup>-methylnicotinamide. In the present study there was a greater excretion of xanthurenic acid during the 3rd week than during the 2nd week, but this was not at the expense of a decrease in N<sup>1</sup>-methylnicotinamide. Moreover, these investigators pointed out that the formation of xanthurenic acid is not directly related to the decreased formation of niacin. Furthermore, they found that the administration of kynurenine, kynurenic acid, and xanthurenic acid to rats on a complete diet failed to produce increased amounts of N<sup>1</sup>-methylnicotinamide in the urine, and concluded, therefore, that the metabolic course from tryptophan to nicotinic acid in the rat is not via the kynurenine pathway. These authors also found that the mechanism for methylating nicotinamide is not impaired in a vitamin B<sub>6</sub>-deficient rat, nor is there an abnormal rate of destruction of N<sup>1</sup>-methylnicotinamide.

Schweigert and Pearson (15) have also reported that when tryptophan is fed pyridoxine-deficient rats and mice have a greatly reduced ability to convert this amino acid to nicotinic acid and N<sup>1</sup>-methylnicotinamide. Their data reveal that when no tryptophan was added to the basal ration (which contributed 3.1 mg of tryptophan per gm) the excretion of N<sup>1</sup>-methylnicotinamide and nicotinic acid in micrograms per rat per day was greater for the animals receiving pyridoxine (93 and 23.4, respectively) than for the animals not receiving it (48 and 9.9), while on the basis of micrograms per gm of food the excretion was not significantly different, 9.3 and 2.4, respectively, for those receiving pyridoxine, and 9.9 and 2.1 for those not receiving it. Thus, on the basal diet pyridoxine had no effect on the conversion of tryptophan to nicotinic acid, 0.14 per cent for the pyridoxine-supplemented group and 0.11 per cent for the pyridoxine-deficient group, while for N<sup>1</sup>-methylnicotinamide the conversion for the pyridoxine-supplemented group was only 0.54 per cent, whereas it was 0.64 per cent for the pyridoxine-deficient group. Only when 100 mg of DL-tryptophan were fed in addition to that in the diet was there a definite

increase in N<sup>1</sup>-methyl nicotinamide and nicotinic acid in favor of the pyridoxine-supplemented group

The explanation for the difference in the results of these investigators (14, 15) and of the present study may lie in the difference in experimental techniques. Their basal rations which contained an adequate amount of tryptophan, 4.5 mg per gm (14) and 3.1 mg per gm (15), were fed *ad libitum*. In the study by the Duke group (14) a complete cessation of growth was reported for the pyridoxine-deficient rats, while in that of the Texas group (15) the deficient animals gained only 10 gm per week, whereas the pyridoxine-supplemented rats grew at a rate of 20 gm per week. Their data indicate that the food consumption of the deficient group was also about half of that for the pyridoxine-supplemented rats.

A definition of nutritional adaptation has been given by Mitchell (34), who has reworded the theorem of Le Chatelier to apply to animal life in its relation to the food supply. "If an animal in equilibrium with its food supply (meaning a well-nourished animal) is subjected to nutritional stress, such as an inadequate (or an excessive) supply of one or more of the essential nutrients, the animal will react in such a way as to minimize, as far as possible, or to undo entirely the effects of the nutritional stress." One of the first and most common adjustments an animal makes to an inadequate diet, regardless of the type of deficiency, is a reduction of food consumption. Experiments intended to determine the specific effect and role of any particular nutrient must be designed to avoid complication by a general response of lowered food consumption and the common effect associated with generalized undernutrition. Equalizing the food intakes of comparative animals by paired feeding differentiates between the specific effect of any particular dietary treatment and the general effect associated with poor appetite. Forced feeding, when feasible, is even more desirable because it not only completely eliminates complication by generalized undernutrition, but also accentuates the inherent effect of any dietary modification by blocking the most common mechanism of adaptation to nutritional stress. Another advantage of forced feeding is a decrease in the time required to produce an acute deficiency, illustrated in this study by the early symptoms and death of the tryptophan-deficient animals.

In studies of the urinary excretion of nicotinic acid and its derivatives it is well to consider that our knowledge of the end-products of nicotinic acid metabolism is incomplete. Perlzweig and Huff (35) have stated that "the excretion of F<sub>2</sub> [N<sup>1</sup>-methyl nicotinamide] represents primarily the resultant of the rates of two or more metabolic reactions involving nicotinic acid methylation to F<sub>2</sub> and the subsequent conversion of F<sub>2</sub> to products as yet unknown." A new metabolite of nicotinamide, identified as the product of oxidation *in vitro* of N<sup>1</sup>-methyl nicotinamide by the quinone

oxidizing enzyme of rabbit liver, has in fact been isolated from human urine after nicotinamide administration, and identified as the 6-pyridone of N<sup>1</sup>-methylnicotinamide (36, 37). Knox and Grossman believe that this pyridone is excreted in an amount at least comparable to that of N<sup>1</sup>-methylnicotinamide. The appearance of a considerable amount of an acid-labile nicotinic acid precursor in the urine of rats receiving large amounts of tryptophan suggests that it may be an intermediate in the synthesis of nicotinic acid (9). Incomplete data obtained in this study indicate that this substance is also produced when only 20 mg of tryptophan are given. The physiologic significance of these compounds, which were not measured in this study, is at present not known.

The present study shows that, while significant differences were observed in urinary excretion values, the only significant difference in gain in weight was that obtained during the 1st week by feeding 2 per cent succinylsulfathiazole. Thus, under the present experimental conditions, there was no relationship between the gain in body weight and the urinary excretion of the compounds tested.

#### SUMMARY

An acid-hydrolyzed casein diet deficient in tryptophan, pyridoxine, and nicotinic acid was fed directly into the stomach. 60 young rats were divided into ten groups of six each. Eight groups provided all possible combinations of the following supplements: 20 mg of L-tryptophan, 0.2 mg of pyridoxine, and 0.2 mg of nicotinic acid. Two groups received 2 per cent succinylsulfathiazole. Individual urinary collections were made for 24 hours during the 2nd and 3rd weeks and after an oral dose of 100 mg of L-tryptophan during the 4th week. The urine was analyzed for free tryptophan, nicotinic acid, N<sup>1</sup>-methylnicotinamide, and xanthurenic acid.

Statistical examination of the data indicated no significant effect of any of the supplements upon gain in weight, even though definite symptoms of pyridoxine and tryptophan deficiency were observed. The presence of 2 per cent succinylsulfathiazole in the diet resulted in a greater gain in body weight during the 1st week, but not in subsequent weeks.

The tryptophan-deficient animals progressively exhibited bloat, diarrhea, and convulsions with screeching. Death occurred as early as the 6th day, and all animals were dead after 13 days of a tryptophan deficiency. Histologically, there was indication of fatty degeneration of the cells in the liver.

The addition of 20 mg of L-tryptophan increased the excretion of nicotinic acid from 78 to 86 per cent, regardless of the presence or absence of pyridoxine or nicotinic acid. The increase in N<sup>1</sup>-methylnicotinamide due to the addition of tryptophan was considerably greater when nicotinic acid was present (204 per cent) than when it was lacking (39 per cent). The

recovery of added nicotinic acid in the form of N<sup>1</sup>-methylnicotinamide was 181 to 198 per cent when 100 mg of L-tryptophan were fed. This indicates that when nicotinic acid was present there was a greater conversion of tryptophan to N<sup>1</sup>-methylnicotinamide than when nicotinic acid was lacking.

Pyridoxine has no significant effect on the conversion of tryptophan to nicotinic acid and N<sup>1</sup>-methylnicotinamide. The presence of nicotinic acid resulted in a greater formation of xanthurenic acid in pyridoxine deficiency.

The results obtained with 2 per cent succinylsulfathiazole in the diet indicate that the conversion of tryptophan to nicotinic acid and N<sup>1</sup>-methylnicotinamide is probably not dependent upon the synthetic activities of intestinal microorganisms.

These observations contribute additional evidence in support of the conclusion that tryptophan is a dietary precursor of niacin as the most logical explanation for the greater urinary excretion of nicotinic acid and N<sup>1</sup>-methylnicotinamide by tryptophan-supplemented animals.

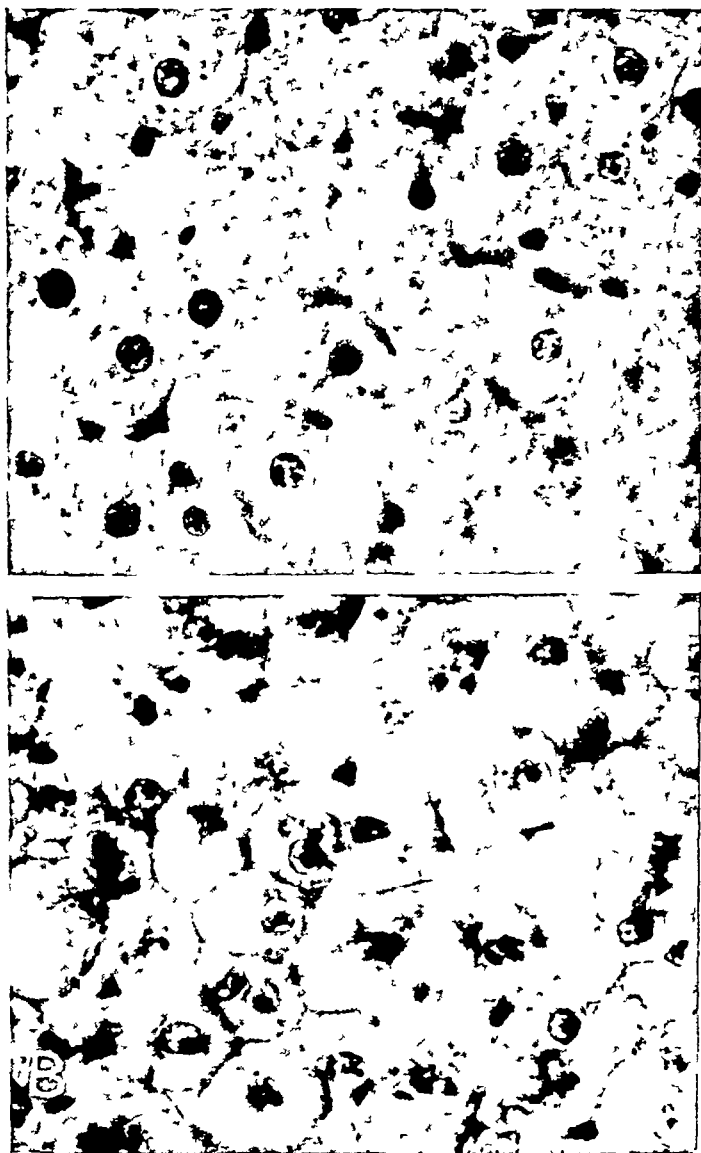
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#### EXPLANATION OF PLATE I

FIG 1 Histological sections of the liver ( $\times 500$ ), hematoxylin-eosin stain of Harris A, normal, B, tryptophan-deficient



(Spector Interrelation of vitamin supplements)



# A MICRO PHOTOFLUOROMETER

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A need arose for a fluorometer sufficiently sensitive to measure the amounts of riboflavin and thiamine in a few hundredths of a ml. of blood or blood serum. There are approximately 1 and 0.25 millimicrograms of riboflavin and thiamine respectively in 20 cmm. of blood serum, an amount conveniently obtainable from the finger. Conventional commercial fluorometers require 100- to 1000-fold greater quantities of these vitamins for adequate measurement. Friedemann and Frazier (1) have reported the use of a more sensitive fluorometer, but the instrument has not been described. Hinton (2) used a very sensitive galvanometer in conjunction with the Spekker fluorometer and was able to measure as little as 1 millimicrogram of thiamine with a reproducibility of 20 per cent. Special capillary cells were employed.

It has been found easily possible to increase the sensitivity (and stability) of a commercial fluorometer 1000-fold by substituting a "multiplier" phototube for the phototube originally present. With this modified instrument, 0.1 millimicrogram of riboflavin ( $10^{-10}$  gm.) in 0.5 ml. of solution can be measured with a precision of 5 per cent. An instrument embodying the same principles is now available commercially.<sup>1</sup>

Description will be given of the changes made to increase the sensitivity and stability of the instrument and of the alterations necessary to decrease the optical blank, the last being more difficult to accomplish.

*Increasing the Sensitivity*—The phototube of the Coleman photofluorometer, model 12, was replaced by an RCA multiplier phototube, No. 1P21.<sup>2</sup> A standard eleven prong tube socket was substituted for the original socket, and was carefully oriented to present the photo surface directly to the

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<sup>1</sup> Farrand Optical Company, Inc., Bronx Boulevard and East 238th Street, New York 66, New York.

<sup>2</sup> There is a series of RCA multiplier phototubes. No. 1P21 is a selected tube of high sensitivity and stability, and is, therefore, rather expensive. No. 931-A, which is a similar but unselected tube, selling for considerably less, is not recommended for the present purpose. As far as the author is aware, none of the other phototubes of the series, like those with other photo surfaces or envelopes, is available in as highly a selected form as No. 1P21.



light beam (The sensitive area on the multiplier tube is only a few mm wide) The eleven well insulated leads from the socket were carried out of the tube housing and instrument box. The tube housing was carefully sealed with black tape to exclude indirect light. Except for the mercury vapor lamp and fan, the original electrical equipment in the box was disconnected and not used.

The electrical circuit employed is very simple (Fig 1). The output of the tube is led without further amplification to the galvanometer (model 34034 of the Rubicon Company, Philadelphia, sensitivity about 1 micro-ampere, full scale). A coarse and fine sensitivity adjustment is provided

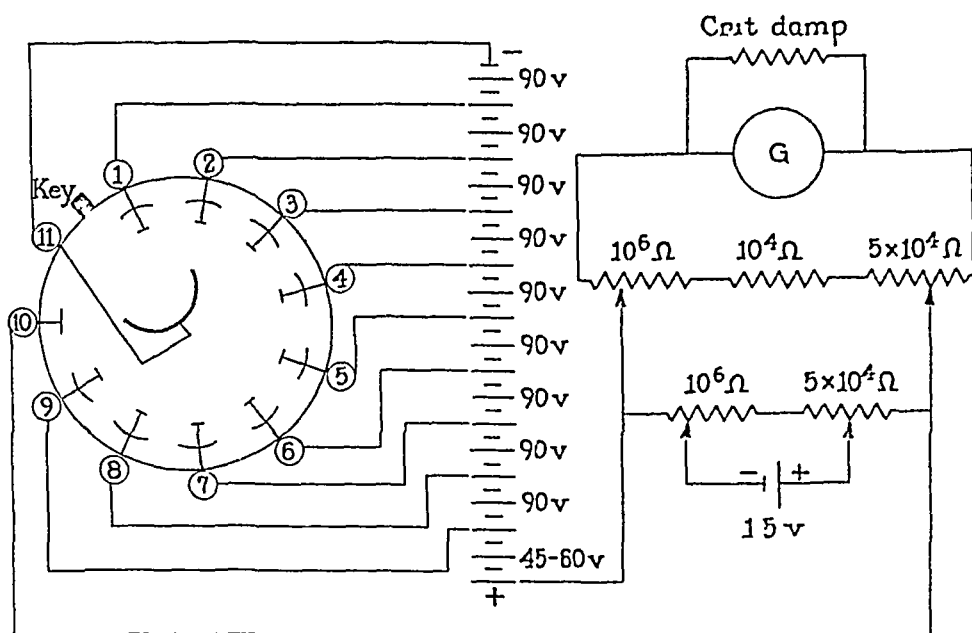


FIG 1 Wiring diagram for multiplier phototube

which permits a 100-fold range in sensitivity. The fixed resistance between the two inexpensive radio potentiometers (Fig 1) is to prevent the sensitivity from being reduced to less than 1 per cent of the maximum. At this lowest sensitivity the galvanometer will go off scale when the phototube output is greater than about 0.1 milliampere, which is a safe upper limit to prevent damage to the tube.

The choice of batteries rather than a transformer for voltage supply is strongly recommended for the sake of simplicity, stability, and freedom from trouble. The voltage must above all be constant. The initial cost of batteries is low and, since the drain is negligible, replacement is only necessary after a year or two of service. Burgess 45 volt B batteries, No W30BPX, and especially Eveready 30 volt Minimax batteries, No 413,

are conveniently small in size, and both have been used successfully. As a precaution, the individual electrodes have been disconnected when not in service, with either gang switches or multiwire cable connectors. The necessity for prevention of electrical leakage with the high voltages used should be taken into account in the construction. However, the inherent design of the phototube and the circuit used is such that electrical leaks are of relatively minor significance in contrast with the troublesomeness of the usual phototube-high impedance circuit.

The battery used for the dark current adjustment may be kept in the circuit continuously, since there is a negligible drain through the high resistance of the shunt.

Because of the great sensitivity of the phototube, it is necessary during readings to cover the tube containing the sample to prevent outside light from entering. Indeed, the external room light must be kept subdued to prevent large galvanometer deflections due to reflections when the tubes are inserted. Red light, to which the phototube is relatively insensitive, may be used conveniently in the room.

*Reduction of Optical Blank*—As the sensitivity of the instrument is increased, the galvanometer deflection with a blank tube of distilled water increases and limits the useful sensitivity obtainable. This blank is chiefly due to fluorescence of the various components of the optical system, *viz*, lenses, light filters and the sample tube itself. Soft glass gives off a weak fluorescence, especially with shorter exciting wave-lengths. This can be largely eliminated by substituting Pyrex tubes. The two original lenses in the instrument also have a slight fluorescence which can be eliminated by replacing them with equivalent Pyrex or quartz lenses. This improvement is, however, insufficient to warrant the trouble under ordinary circumstances. It is more difficult to eliminate fluorescence of the light filters. The effect of their fluorescence can, however, be reduced by reducing the scattered light within the system. Two changes proved helpful in this regard. In the path of the exciting light beam was fixed a wooden baffle which was so carved that only the central portion of the tube was illuminated (Fig 2). The wood was blackened with India ink. To reduce scattered light further, the inner chamber which surrounds the sample tube was lined with black cloth, which is much less reflecting than the original smooth, blackened metal surface. After these changes were made, the optical blank was measured with the light filter combination appropriate for riboflavin. (The primary filter, Coleman B<sub>2</sub>, consists of Corning glass Filters 5113 and 3389, the latter facing the light source. The secondary filter, Coleman PC2, is Corning glass Filter 3486.) A 19 mm diameter soft glass tube filled with redistilled water gave a galvanometer deflection equivalent to that obtained with 3 millimicrograms of riboflavin in 8 ml

By substituting a Pyrex tube, the blank was reduced to just half of this value

The ratio of riboflavin reading to optical blank was still further improved by reducing the sample volume and using smaller Pyrex tubes (9 mm outside diameter  $\times$  10 cm) in an adapter (Fig 2) With this arrangement, which required no more than 0.5 ml of solution, the optical blank was equivalent to only 0.2 millimicrogram of riboflavin in per 0.5 ml The reduc-

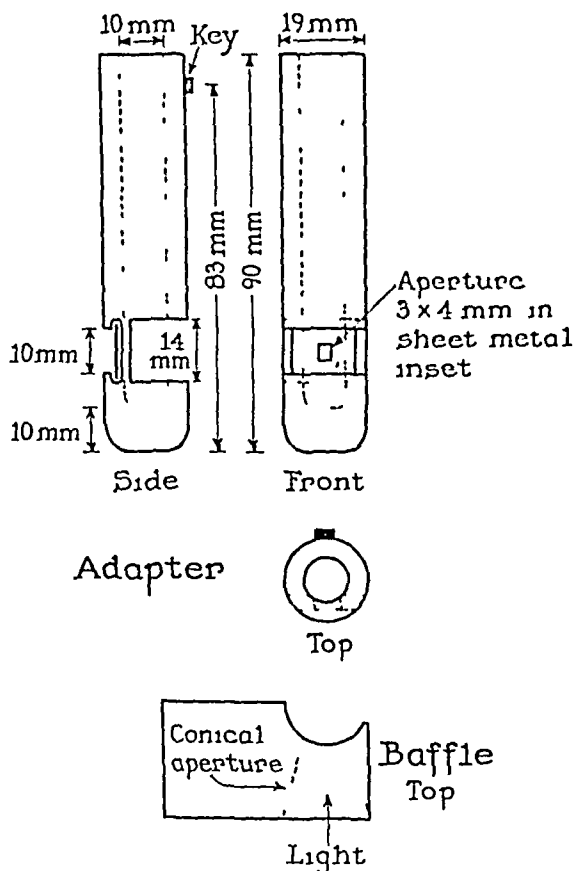


FIG 2 Adapter and baffle for micro photofluorometer

tion in volume has the further general advantage of increasing the concentration of measured substances relative to the blank fluorescence of the reagents used

Greater difficulty was encountered in reducing the optical blank with the filter combination used for the measurement of thiochrome, and other substances requiring the 365 m $\mu$  mercury band for excitation (the condensation products of diphosphopyridine nucleotide or N<sup>1</sup>-methylnicotinamide with ketones (3), pyridoxime derivatives (4), xanthopterin and related products, quinine, etc) The usual filter combination consists of Corning

glass Filter 587 as the primary (Coleman, B1) and Corning Filters 3389 and 4308 as the secondary (Coleman, PC1) The Corning Filter 3389 is itself quite fluorescent This can be decreased by inserting a film of Wratten gelatin filter, No 2A (Eastman Kodak Company), between Filters 3389 and 4308 with Filter 3389 faced toward the phototube Filter 2A, gelatin film, prevents the ultraviolet light from reaching Filter 3389

TABLE I  
*Riboflavin\* Measurements with Micro Photofluorometer*

8 ml volume		0.5 ml volume	
Riboflavin present	Riboflavin found	Riboflavin present	Riboflavin found
<i>millimicrograms</i>	<i>millimicrograms</i>	<i>millimicrograms</i>	<i>millimicrograms</i>
0 53	0 54	0 106	0 107
0 53	0 59	0 106	0 105
0 53	0 55	0 106	0 108
		0 106	0 106
Average 0 53	0 56	0 106	0 106
1 99	2 00	0 53	0 54
1 99	2 00	0 53	0 52
1 99	2 07	0 53	0 51
		0 53	0 51
Average 1 99	2 02	0 53	0 52
20 8	20 7	1 99	1 98
20 8	20 9	1 99	2 04
20 8	21 0	1 99	2 14
		1 99	2 05
Average 20 8	20 9	1 99	2 05

\* The riboflavin was measured in the usual manner by comparing the initial fluorescence with the increase in fluorescence when an internal riboflavin standard was added

Unfortunately, Filter 2A is itself slightly fluorescent, apparently owing to the gelatin, hence, the combination is still imperfect although much improved So modified, the optical blank with 0.5 ml of redistilled water in a 9 mm Pyrex tube was equivalent to the fluorescence of the thiochrome from 0.6 millimicrogram of thiamine in 0.5 ml Although this is not as low a blank as is desirable, the fluorescence of reagents and other substances likely to be present is larger than the optical blank, which becomes, therefore, of secondary importance

*Performance of Micro Fluorometer*—Table I illustrates the measurement of 0.1, 0.5, and 2 millimicrogram quantities of riboflavin in a volume of 0.5 ml, and 0.5, 2.0, and 20 millimicrogram quantities in 8 ml. The precision of measurement is seen to be quite adequate, and compares favorably with that of macro instruments which require 1000-fold larger samples. At full sensitivity, with the adapter, 0.1 millimicrogram of riboflavin in 0.5 ml (1 part in 5 billion) gave a galvanometer deflection of 6 divisions. At one-third sensitivity, without the adapter, 0.5 millimicrogram of riboflavin in 8 ml (1 part in 16 billion) gave a deflection of 8 divisions. Thus, if the optical blank could be further reduced, it would be possible to measure riboflavin at a dilution of 1 to 50 billion. The response with thiochrome is nearly 20 times greater than with riboflavin, hence, it could conceivably be measured at a dilution of 1 to a trillion if the optical blank were sufficiently reduced.

#### DISCUSSION

Fluorimetry is inherently a much more sensitive analytical tool than colorimetry for measuring *concentrations* of substances. In colorimetric procedures a significant percentage of the light must be absorbed, and this is determined almost entirely by the concentration of the substance and the length of light path. In measurement of a fluorescent substance the sensitivity is proportional not only to the concentration of the substance and the length of light path, but also to the intensity of illumination and the sensitivity of the photometer. There are limitations to the increase in illumination permissible with light-sensitive materials, but the sensitivity of the photometer can be greatly increased. There are few substances which can be measured colorimetrically at concentrations less than 1 part per 10 million with a reasonable light path. This is a concentration 2000 times stronger than that of the riboflavin solution measured above, and this vitamin is not an exceptionally fluorescent substance.

#### SUMMARY

A micro fluorometer is described which is 100 to 1000 times more sensitive than existing commercial instruments, and which is capable of measuring as little as 0.1 millimicrogram of riboflavin, for example, with a precision of 5 per cent, and larger quantities with at least the precision attainable with macro fluorometers.

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# A SIMPLE QUANTITATIVE CHEMICAL METHOD FOR ESTIMATING $\gamma$ -GLOBULIN IN HUMAN SERUM\*

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Both sodium sulfate and potassium phosphate have been employed for the quantitative estimation of globulin fractions in human serum (1-6). With these procedures, the quantities of the various globulins are determined by subtractive methods after precipitation of portions of total globulin. Also the volumes of salt solution added per ml of serum are large. The method outlined here for estimation of  $\gamma$ -globulin departs from these procedures in that it entails the direct estimation of the protein content of the precipitate obtained by adding a small volume of saturated ammonium sulfate to undiluted serum. Certain characteristics of the fraction precipitated have been examined. In both normal and pathological sera, the quantity of protein present in this fraction was found to correlate well with values estimated electrophoretically for  $\gamma$ -globulin in the same samples.

## Method

### Reagents—

- 1 Saturated ammonium sulfate brought to pH 7.0 with ammonium hydroxide
- 2 0.33 saturated ammonium sulfate (pH 7.0)
- 3 The dilute biuret reagent of Weichselbaum (7)

*Technique*—To 1.0 ml of fresh serum in a 12 ml centrifuge tube is added 0.5 ml of saturated ammonium sulfate drop by drop, the precipitate being shaken *thoroughly* after addition of each 0.1 ml. The precipitate generally will disappear after shaking until more than 0.3 ml of salt solution is added. The suspension is placed at 4° overnight. It is then centrifuged for 30 minutes at 3000 R P M in an angle centrifuge. The pale yellow, clear supernatant fluid is removed by aspiration and discarded. The packed precipitate is *finely emulsified* in the tube with a stirring rod after addition of 3.0 ml of 33.3 per cent saturated ammonium sulfate. The tube is re-centrifuged for 30 minutes at 3000 R P M and the clear, colorless supernatant removed. The precipitate is dissolved by stirring and shaking in 10 ml of

\* Aided by grants from the Physicians' Research Fund of the University of Utah School of Medicine, the Fluid Research Fund of the Rockefeller Foundation, and the Life Insurance Medical Research Fund.

0.85 per cent sodium chloride. To 5.0 ml of this slightly opalescent colorless fluid are added 5.0 ml of the biuret reagent. The protein content is measured in a Coleman model J1 universal spectrophotometer, with 7 mm cuvettes and at a wave-length setting of 555 m $\mu$ .

Except for the initial refrigeration of the precipitate, all steps are performed at room temperature. If the serum is centrifuged immediately after the initial addition of ammonium sulfate, the protein content of the precipitate frequently is 10 to 20 per cent lower than that of a sample permitted to stand overnight in the refrigerator. When the serum has a high lipid content, the precipitate redissolved in saline may appear turbid even after the biuret reagent has been added. In such instances the lipid is extracted with ether immediately before the spectrophotometric reading.

Repeated protein determinations on a standard solution of rabbit serum with the biuret technique and with a micro-Kjeldahl method (8) gave agreement within  $\pm 2$  per cent. The small amount of ammonium sulfate present in the precipitate redissolved in saline was not found to affect significantly the development of the biuret color reaction. In a few experiments in which sera were kept frozen for as long as 4 weeks, the quantities of precipitate were equal to those obtained with fresh sera.

### *Experimental Observations*

*Accuracy of Method*—The quantity of washed precipitate (Fraction G G-33.3) obtained with this technique was determined in triplicate on the sera of ten normal subjects (Table I). The standard deviation of the method was 0.021 gm, which gave a coefficient of variation of  $\pm 2.7$  per cent.

*Protein-Bound Phosphorus Content*—Aliquots of protein obtained by dialysis of whole serum, of the supernatant fraction after precipitation with 33.3 per cent saturated ammonium sulfate, and of the washed 33.3 per cent precipitate were digested with perchloric acid (9) and the phosphorus content determined spectrophotometrically (10). With the mean values obtained from determinations on seven separate sera and then fractions, the protein-bound phosphorus content, as mg of P per gm of protein, is as follows: whole serum 2.02, supernatant 1.75, and washed precipitate 0.24. The protein-bound phosphorus content of the precipitate, while low, is somewhat greater than that of electrophoretically isolated  $\gamma$ -globulin as determined by Blum and coworkers (11).

*Antibody Content*—Enders (12) has shown that many of the antibodies present in human sera are concentrated in Cohn's Fraction II, which consists almost entirely of  $\gamma$ -globulin (13). A few antibodies, however, such as those to the typhoid O antigen and isoagglutinins to blood types A and B were found to be concentrated in Fraction III<sub>1</sub>, which consists mostly of  $\beta$ - and  $\gamma$ -globulins. Using methods of antibody measurement similar to

those of Enders, we determined the concentration in whole serum and in the two fractions obtained with ammonium sulfate. Each fraction after

TABLE I

*Triplicate Determinations of 33.3 Per Cent Saturated Ammonium Sulfate Precipitate (Washed) in Ten Normal Human Sera*

Expressed in gm of precipitate per 100 ml of serum

Serum	Determination 1	Determination 2	Determination 3	Mean
R B	0 661	0 666	0 663	0 665
B G	0 846	0 828	0 850	0 841
R G	1 052	1 210	1 104	1 132
V D	0 752	0 742	0 718	0 737
H A	0 980	0 994	1 000	0 991
M N	1 070	1 103	1 070	1 081
V J	0 738	0 722	0 738	0 733
B L	1 037	1 055	1 055	1 049
J T	0 679	0 661	0 668	0 669
A D	1 077	1 045	1 037	1 053

Standard deviation of the method, 0 021 gm, coefficient of variation, =2 66 per cent

TABLE II

*Antibody Content in Whole Serum and in Serum Fraction*

The antibody content of each fraction was determined by using a protein content per ml identical with that which this fraction possessed in the original serum. Antibody titers are expressed as reciprocals of the last dilution of serum or fraction giving 2+ agglutination with the exception of diphtheria antitoxin which is measured by the rabbit skin test, the values being in units per ml.

Antibody	Source	Antibody concentration per ml.		
		Whole serum	Fraction G G.33.3	Supernatant
Diphtheria antitoxin	Pooled normal sera		0 075	0 018
" "	Convalescent patient	0 32	0 17	0 14
Typhoid H	Immunized subject	5120	5120	2560
" O	" "	160	0 (<20)	80
Isoantibody against group A cells	Normal subject	64	0 (<8)	64
Heterophil antibody	Patient with infectious mononucleosis	320	80	320

dialysis was brought to the protein content it possessed in the original serum. The results (Table II) indicate that diphtheria antitoxin<sup>1</sup> and

<sup>1</sup> Diphtheria toxin and antitoxin were furnished by the Lederle Laboratories Division, American Cyanamid Company



typhoid H antibody are concentrated in Fraction G G 33 3. However, isoagglutinins to group A cells, the heterophil antibody, and typhoid O antibody were not concentrated in this fraction.

*Electrophoretic Composition of Fractions*—Analyses were made in a Tiselius apparatus equipped with a Longworth schlieren scanning device.

TABLE III

*Electrophoretic Analysis of Whole Serum and of Fractions Obtained with 33 S  
Per Cent Saturated Ammonium Sulfate*

The values are expressed in gm of protein per 100 ml of serum and as per cent of the total serum protein.

Sample	Total protein	Albumin	Globulin						
			$\alpha_1$ and $\alpha_2$		$\beta_1$ and $\beta_2$		$\gamma_1$ and $\gamma$		
Pooled normal serum									
	gm	gm	per cent	gm	per cent	gm	per cent	gm	per cent
Whole serum	7.79	4.60	59	1.17	15	0.93	12	1.09	14
Supernatant*	5.91	4.20	71	1.00	17	0.59	10	0.12	2
Fraction G G 33 3	1.04	0	0	0.17	16	0.11	11	0.76	73
Wash of Fraction* G G 33 3	0.84	0.21	25	0.19	22	0.06	7	0.39	46
Whole serum	7.67	4.75	62	1.07	14	0.84	11	0.96	13
Supernatant*	6.42	4.43	69	1.09	17	0.90	14	0	0
Fraction G G 33 3	0.97	0.01	1	0.05	5	0.13	14	0.78	80
Wash of Fraction* G G 33 3	0.28	0.09	31	0.03	12	0.03	12	0.13	45
Serum from case of arthritis									
Whole serum	7.92	3.64	46	1.19	15	0.79	10	2.30	29
Supernatant*	5.20	3.27	63	1.35	26	0.57	11	0	0
Fraction G G 33 3	2.07	0	0	0.06	3	0.29	14	1.72	83
Wash of Fraction* G G 33 3	0.65	0.23	35	0	0	0.08	12	0.34	53

\* Corrected for the volume increase resulting from the addition of ammonium sulfate solution.

Runs were made at 1.5°, with a veronal buffer at pH 8.4 to 8.6, an ionic strength of 0.1, and a cell protein concentration of 1.5 gm per cent. In the various studies here and in the subsequent section, the mobilities of the various fractions exhibited the following ranges ( $\times 10^{-5}$  sq cm per volt per second):  $\gamma$ -globulin, 0.8 to 1.3,  $\beta$ -globulin, 2.8 to 3.7,  $\alpha$ -globulin, 4.1 to 5.5, albumin, 6.0 to 6.7.<sup>2</sup>

<sup>2</sup> Electrophoretic studies were carried out by Dr. Emil L. Smith with the technical assistance of D. M. Brown at the Laboratory for the Study of Hereditary and Metabolic Disorders, University of Utah School of Medicine.

In Table III, the electrophoretic composition of the whole serum and of the two fractions obtained with ammonium sulfate, as well as of the wash fluid, are presented. Fraction G G 33.3 was relatively homogeneous, being 73 to 83 per cent  $\gamma$ -globulin. By simple calculation, it may be shown that Fraction G G 33.3 contains from 70 to 82 per cent of the total  $\gamma$ -globulin present in whole serum. Most of the remainder is lost during the process of washing the precipitate.

*Comparison of Chemical and Electrophoretic Values for  $\gamma$ -Globulin*—In thirty-seven human sera (seven from normal subjects and thirty from patients with various diseases) the numerical value for  $\gamma$ -globulin as determined chemically was found to correlate reasonably well with the value for

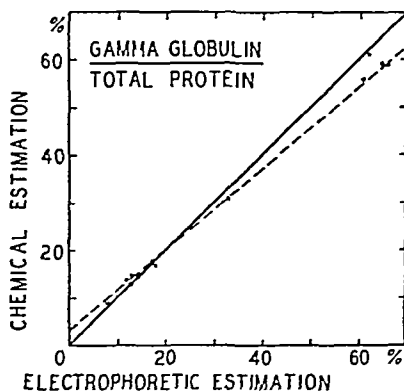


FIG. 1. The solid line would obtain if perfect correlation between chemical and electrophoretic methods for estimation of  $\gamma$ -globulin to total protein had occurred. The dotted line is derived from calculations to fit the observed points, assuming the electrophoretic estimations to be true values. Because of overlapping, the entire thirty-seven values cannot be represented graphically.

$\gamma$ -globulin obtained by electrophoretic estimation (Fig. 1). The values are expressed as per cent of the total serum protein. The standard deviation of the chemical values from the electrophoretic values was 2.01 per cent, with a coefficient of variation of 10 per cent. The mean electrophoretic value for  $\gamma$ -globulin in the thirty-seven samples of sera was 17.9 per cent. A curve constructed to fit the points in Fig. 1 has the formula  $y = 3.419 + 0.8498x$ . The value of  $S^2$  is 4.72.<sup>3</sup> The numerical values for  $\gamma$ -globulin (Fraction G G 33.3) determined chemically are somewhat high in the normal range and somewhat low in the high range for corresponding values of  $\gamma$ -globulin determined electrophoretically.

*Solubility Studies*—The precipitation of a  $\gamma$ -globulin fraction from undi-

<sup>3</sup>  $S^2 = (\sum (y - \bar{y})^2) / (n - 2)$ , where  $y$  = the observed value and  $\bar{y}$  = the calculated value.

luted serum with saturated ammonium sulfate may be criticized because of the likelihood of occlusion of other proteins in the precipitate and because of the greater tendency for lipide-rich  $\beta$ -globulin to adhere to the precipitate in undiluted serum compared with diluted serum (14). However, with dilution of serum prior to precipitation, the recovery becomes poor, particularly in those sera in which the  $\gamma$ -globulin fraction is not increased.

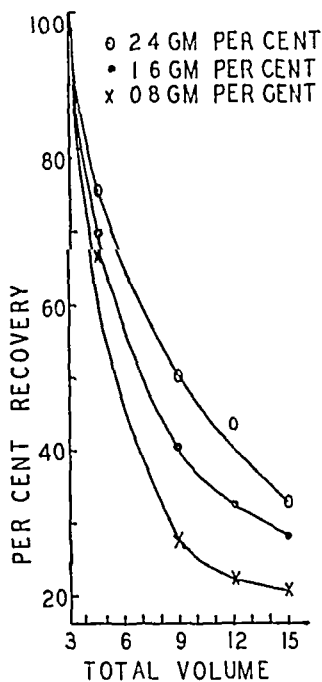


FIG 2

FIG 2 Quantity of protein recovered in the precipitate (as per cent of protein quantity introduced) ( $y$ ) with increasing volumes of 33.3 per cent saturated ammonium sulfate solution ( $x$ ) for three separate amounts of Fraction G G 33.3

FIG 3 Effect of dilution upon the quantity of precipitate recovered for four specimens of whole serum in which increasing concentrations of Fraction G G 33.3 were demonstrated by the usual method. Percentage recovery with increasing dilution is arbitrarily expressed as per cent of the protein precipitate obtained with a 4.5 ml volume of salt solution.

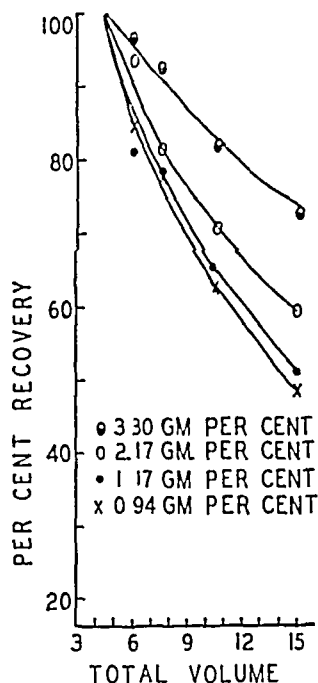


FIG 3

A large quantity of washed and dialyzed Fraction G G 33.3 was prepared from pooled normal human serum by the method outlined above. 10 ml quantities of a given concentration of protein solution were diluted with increasing volumes of saline prior to addition of sufficient saturated ammonium sulfate to produce 33.3 per cent saturation. In each instance the precipitate was washed with 30 ml of 33.3 per cent saturated ammonium sulfate. The total volumes of solution in contact with each protein concentra-

tion ranged from 4.5 ml for undiluted protein solution (1 ml of protein solution + 0.5 ml of salt + 3.0 ml of wash fluid) to 15 ml (dilution with 7 ml of NaCl). These procedures were carried out for protein concentrations of 2.40, 1.60, and 0.80 gm per cent. The quantity of protein in the precipitate was measured and was expressed as per cent of the original protein concentration of the solution. As is seen in Fig 2, the greater the total volume of solution in contact with protein, the less the amount of protein that may be expected to precipitate. Moreover at any given volume, the percentage recovery of the protein introduced becomes less with decreasing concentrations of protein in the original solutions. Similar results were obtained with a sample of Cohn's Fraction II (13). This fraction, however, was far more soluble in 33.3 per cent saturated ammonium sulfate than was ours.

In Fig 3 is illustrated a similar study on normal sera and pathologic sera in which Fraction G G 33.3 content was increased. Here the amount of precipitate present with the usual procedure (4.5 ml of the total volume) is taken to represent 100 per cent yield and the precipitate recovered in diluted serum is expressed in relation to this amount in per cent. While the percentage recovery with dilution fell off steeply for sera with normal values for Fraction G G 33.3, this was not the case for the pathologic sera, in which this fraction was increased. The similarity of these results with whole sera to those obtained in solutions of Fraction G G 33.3 is evident.

#### DISCUSSION

Because of the overlapping solubility characteristics of the various globulin fractions, there is no simple salting-out procedure which will permit quantitative recovery of a homogeneous  $\gamma$ -globulin. With the usual salting-out methods for globulin fractionation, the volume of salt solution added per ml of serum is large. As is indicated by our solubility studies, such methods, if scaled to yield a homogeneous fraction of  $\gamma$ -globulin, will give a poor recovery when the  $\gamma$ -globulin content is normal or moderately increased and a relatively greater recovery when this constituent is considerably increased (2). Such methods adjusted to give recoveries of protein *numerically* equivalent to the electrophoretic content of  $\gamma$ -globulin in *normal* serum necessarily must involve the simultaneous precipitation of considerable amounts of other globulin fractions. The method that we have presented, while it does not yield a pure fraction of  $\gamma$ -globulin, permits sufficient recovery to detect relatively slight quantitative changes in the ranges in which this constituent is normal or moderately elevated as well as in the range in which it is greatly increased.

## SUMMARY

A simple method is described for measuring the amount of protein precipitated from undiluted serum to which is added sufficient ammonium sulfate to produce 33.3 per cent saturation.

Electrophoretic studies indicate that this fraction consists mainly of  $\gamma$ -globulin and demonstrate a good numerical correlation between electrophoretic values for  $\gamma$ -globulin and chemical values in normal and abnormal sera.

Investigation of some solubility characteristics of this fraction reveals certain advantages in making the precipitation from undiluted rather than diluted serum.

Dr. G. R. Greenberg suggested this method to us. Dr. Emil L. Smith and Dr. M. M. Wintrobe gave helpful criticism.

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# THE ACTION OF ENZYMES ON PARAMECIN\*

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Paramecin, the "killer" substance of *Paramecium aurelia* (stock 51, variety 4), is easily inactivated under mild conditions, as is evidenced by the influence of the hydrogen ion concentrations on its stability (2). Paramecin is most stable at a hydrogen ion concentration of 8.0 to 8.5, nevertheless, 13 per cent of the activity is lost after an incubation of an hour at 30.0° at this pH. The heat of activation of the inactivation reaction, calculated from the reaction rates at 30° and 40°, was found to be 126,000 calories per mole at pH 7.0, a value typical for either an enzyme or a protein or both.

The analysis of paramecin by means of enzyme digestion experiments which seek to identify specific substances and linkages by specific enzymes might reveal some of the chemical nature of this compound. Methods of this kind have been invaluable in the analysis of the chemical nature of the chromosome by van Herwerden (3), Caspersson (4), Mazia (5), and Catchside (6), and in studies by Brachet (7) on the rôle of nucleic acids during embryonic development. It could not be hoped that the application of the enzyme digestion technique would specifically identify paramecin as a chemical entity, however, valuable information might be obtained on the presence in the paramecin molecule of certain classes of compounds which are essential for its biological activity.

## Methods and Results

*Enzyme Preparations*—The action of the following enzyme preparations on paramecin was investigated: (a) lysozyme in the form of its crystalline carbonate prepared by Alderton and Fevold (8), (b) a lysozyme preparation, MI35A, prepared by Meyer and Hahnel (9), (c) hyaluronidase, Preparation HD88-99, 35 viscosity reducing units per mg, prepared according to the procedure of Madnaveta (10) and obtained from Dr. Erwin Schwenk, (d)

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Part of this study was presented in a symposium, "Plasmagenes, genes and characters in *Paramecium aurelia*," at the 114th meeting of the American Association for the Advancement of Science, Chicago, Illinois, December 26-31, 1947. A preliminary report is in press (1).

crystalline trypsin, obtained from Armour and Company, (e) papain, purified according to the procedure of Irving *et al* (11), (f) pepsin (Merck and Company, Inc.), (g) crystalline chymotrypsin, obtained from Armour and Company, (h) crystalline ribonuclease (Dr Kunitz), (i) crystalline desoxyribonuclease (Dr McCarty), and (j) protein B, a proteolytic enzyme isolated from beef pancreas (12), obtained from Dr Laskowski.

We want to express our deep appreciation to Dr G Alderton and Dr H L Fevold of the Western Regional Research Laboratory, Dr K Meyer of Columbia University, Dr Edwin Schwenk of the Schering Corporation, Dr M Kunitz and Dr M McCarty of The Rockefeller Institute for Medical Research, and to Dr M Laskowski of Marquette University for their more than generous gifts of several of the above enzyme preparations.

*Method of Testing*—A detailed description of the methods of cultivating *Paramecium aucta* and of the procedure of testing the activity of paramecin extracts has been published (2). The action of the enzymes was determined by incubating the paramecin extract with the particular enzyme under investigation at 30.0°, while care was being taken in each case to adjust the substrate in such a manner that optimal activity for the enzymic action was assured. Samples were taken after temperature adjustment and at different time intervals up to 60 minutes. After dilution the samples were tested in the usual way for activity, by distributing 1 cc. of the dilution to ten depression slides containing sensitive animals (*Paramecium aucta*, stock 31, variety 8). After incubation for 48 hours at 27° the dead and affected animals were counted. The actual counts were recalculated on a percentage basis. A control in phosphate buffer of pH 7.0 and a control in which the substrate had the same composition as the one used in the enzyme experiment, but in which the enzyme was replaced by a buffer solution, were included in each experiment. All enzymes were tested in the concentration of the final dilution for any action on sensitive paramecia. In no instance could any lethal effect or paramecin activity of the various enzymes be detected. All the data given are averages of at least duplicate determinations.

*Lysozyme*—Lysozyme activity has an optimum at pH 5.3 when the activity is measured viscosimetrically. At this hydrogen ion concentration paramecin is very rapidly inactivated. However, a second optimum can be demonstrated at pH 7.0 when lysozyme activity is measured by the increase in reducing groups in the substrate. The depolymerase action of lysozyme at this pH is only slightly less than at its optimum. The addition of sodium chloride in a concentration of 0.2 M is necessary to insure highest activity of the depolymerizing action of lysozyme, while the opening of glucosidic linkages does not seem to require halogen (9). It was decided therefore to investigate the action of lysozyme on paramecin in the presence of sodium

chloride It is clear from Table I that the two lysozyme preparations had no effect on the activity of paramycin No significant difference is apparent between the inactivation in the absence and in the presence of the enzyme It should be noted that paramycin is much more unstable in the presence of 0.2 M sodium chloride than when sodium chloride is omitted from the substrate

TABLE I  
*Action of Lysozyme on Crude Paramycin Extracts*

Samples of 0.05 cc were taken at 30 and 60 minutes and diluted 1:500 before 1 cc of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours. The buffer added to Control 2 was 0.4 M in NaCl. All of the lysozyme preparations were dissolved in phosphate buffer of pH 7.0, which contained 0.4 M NaCl. The final concentration of NaCl was 0.2 M in every experiment, except Control 1, which contained no salt.

	Composition of medium	Per cent activity		
		0 min	30 min	60 min
Control 1	1.0 cc paste	100.0	72.3	45.2
" 2	1.0 " buffer			
	1.0 " paste	100.0	47.8	20.4
" 3	1.0 " buffer, 0.2 M NaCl			
	1.0 " lysozyme, 200 $\gamma$ per cc	0	0	0
	1.0 " buffer			
Lysozyme MI35A,	1.0 " paste	100.0	45.9	21.2
1 $\gamma$ per cc	1.0 " lysozyme, 2 $\gamma$ per cc			
Lysozyme MI35A,	1.0 " paste	100.0	44.8	19.3
10 $\gamma$ per cc	1.0 " lysozyme, 20 $\gamma$ per cc			
Lysozyme MI35A,	1.0 " paste	100.0	46.2	22.0
100 $\gamma$ per cc	1.0 " lysozyme, 200 $\gamma$ per cc			
Lysozyme W R, *	1.0 " paste	100.0	40.1	19.0
1 $\gamma$ per cc	1.0 " lysozyme, 2 $\gamma$ per cc			
Lysozyme W R, *	1.0 " paste	100.0	43.5	18.0
10 $\gamma$ per cc	1.0 " lysozyme, 20 $\gamma$ per cc			
Lysozyme W R, *	1.0 " paste	100.0	49.2	22.3
100 $\gamma$ per cc	1.0 " lysozyme, 200 $\gamma$ per cc			

\* Western Regional Research Laboratory

*Hyaluronidase*—The presence of hyaluronidase in pathogenic bacteria (13-16) and spermatozoa (17-19) suggests that this enzyme plays a rôle in the processes of invasion by the depolymerization of the mucoid ground substance of connective tissue. Evidence has also been presented that hyaluronidase and the "spreading factor," which increases the permeability of the host tissue, are identical (20-22). Paramycin extracts were incubated with hyaluronidase in different concentrations. The results, which



are reported in Table II, indicate that hyaluronidase is without any effect on the activity of paramecin

*Papain*—Papain was purified according to the procedure of *Irving et al* (11). The final precipitate was dried *in vacuo*. After it was equilibrated with the moisture of the laboratory atmosphere, it contained 12.79 per cent Kjeldahl N, and had about the same activity as the product obtained by the above workers. Hoover and Kokes (23) reported that the initial rate of digestion by papain has an optimum at pH 7. Although the optimum for final digestion of proteins is at pH 5, it was decided to test papain at a pH

TABLE II

*Action of Hyaluronidase on Crude Paramecin Extracts*

The enzyme was dissolved in phosphate buffer, pH 7.0. Samples were taken at the designated time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min	20 min	40 min	60 min
Control 1	1.0 cc. paste	100.0	79.7	65.5	42.0
" 2	1.0 " buffer, pH 7.0				
	1.0 " hyaluronidase, 20 mg per cc	0	0	0	0
Hyaluronidase, 10 mg per cc	1.0 cc. paste	100.0	76.0	63.7	45.9
	1.0 " hyaluronidase, 20 mg per cc				
Hyaluronidase, 1 mg per cc	1.0 cc. paste	100.0	77.0	63.6	45.0
	1.0 " hyaluronidase, 2 mg per cc				
Hyaluronidase, 0.1 mg per cc	1.0 cc. paste	100.0	81.0	62.8	41.5
	1.0 " hyaluronidase, 0.2 mg per cc				

of 7, since there seems to be no essential difference in the attack of the enzyme on proteins. The enzyme was activated by incubating 25 mg. of the purified preparation in 25 cc. of phosphate buffer (pH 7.0) with an equal amount of cysteine for 1 hour at 30.0°. It can be seen from Table III that papain did not inactivate paramecin.

*Trypsin*—Apparent inactivation to about 20 per cent of the original activity took place within the first 20 minutes when paramecin extracts were incubated with trypsin. Hereafter no further inactivation took place. This behavior suggested that paramecin formed an inactive complex with trypsin, similar to those formed by tobacco mosaic virus and trypsin, as reported by Stanley (24), Loring (25), and Kleczkowski (26). In order to

test this, 4 cc of a paramycin extract were incubated with 1 mg of trypsin for 20 minutes at 30.0°. This suspension was then tested without dilution and in increasing dilutions (Table IV). Apparently trypsin inactivates paramycin by reversible complex formation. The complex dissociates easily upon dilution.

TABLE III

*Action of Papain on Crude Extracts of Paramycin*

Samples were taken at the designated time intervals and diluted 1:500 before testing. 1 cc of the dilution was distributed over ten depressions containing sensitive *Paramoecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min	20 min	40 min	60 min
Control 1	1.0 cc paste	100.0	76.0	60.0	47.0
" 2	1.0 " buffer, pH 7.0				
	1.0 " paste	100.0	97.0	96.0	94.0
	1 cc buffer, pH 7.0				
Papain	1 mg cysteine per cc				
	1.0 cc paste	100.0	98.5	95.5	92.5
	1 cc buffer, pH 7.0				
	Papain, 1 mg per cc				
	Cysteine, 1 mg per cc				

TABLE IV

*Dissociation of Inactive Complex of Paramycin with Trypsin on Dilution*

	Dilution	Nos. of sensitive <i>Paramoecia</i> killed per cc of diluted paste	Total activity
Control Trypsin	1:10,000	61	610,000
		96	96
	1:100	764	76,400
	1:1,000	127	127,000
	1:10,000	57	570,000
	1:100,000	5	500,000

*Pepsin*—Stanley (27) reported that tobacco mosaic virus was inactivated when incubated with pepsin under conditions favorable for optimal proteolytic activity of pepsin. The optimal pH for the proteolytic activity of pepsin is 2.0, a hydrogen ion concentration at which paramycin is instantaneously inactivated (1). The lowest hydrogen ion concentration at which different rates of inactivation could be determined with any degree of accuracy was that of pH 6. At this pH pepsin still exerts proteolytic

activity (28) It was found (Fig 1) that the presence of pepsin in the substrate caused a greater inactivation than that found in the control

*Chymotrypsin*—Crystalline chymotrypsin was dissolved in a phosphate buffer of pH 8 The solution contained 2 mg of chymotrypsin per cc

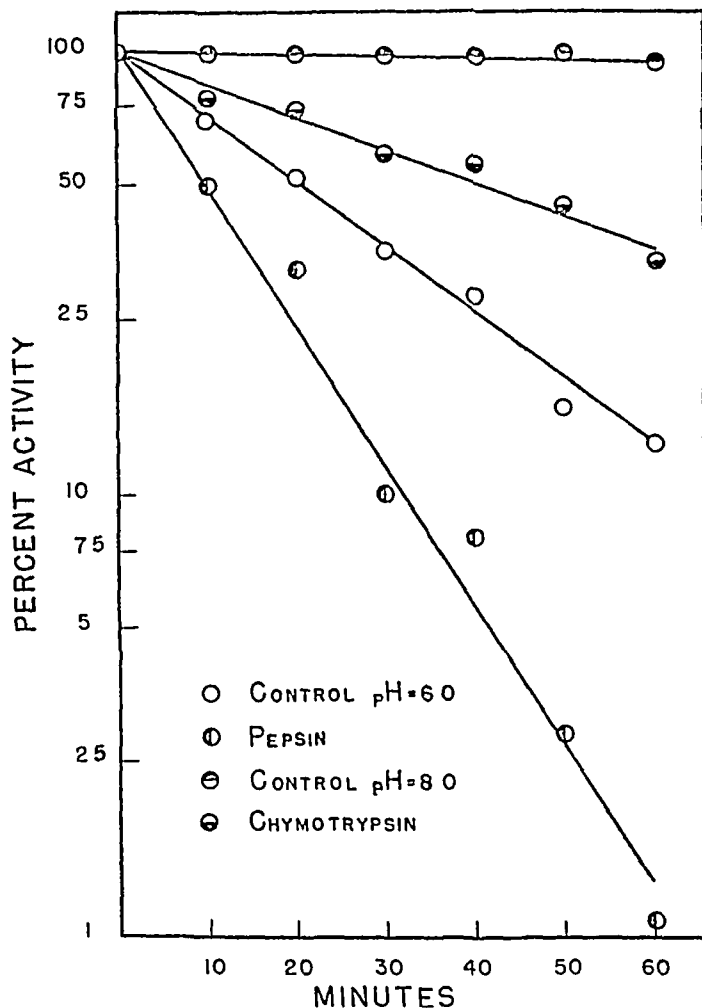


FIG 1 Inactivation of paramycin by pepsin and crystalline chymotrypsin Pepsin, 1 cc of paramycin paste phosphate buffer (pH 6.0) was incubated with 1 cc of a pepsin solution (Merck, U S P, granular, 2 mg per cc in phosphate buffer, pH 6.0) at 30°, chymotrypsin, 1 cc of *Paramycin* paste in phosphate buffer (pH 8.0) was incubated with 1 cc of a chymotrypsin solution (Armour and Company crystalline, 2 mg per cc in phosphate buffer, pH 8.0) at 30°

Incubation of a paramycin paste with this chymotrypsin solution resulted in a greater loss of activity than in the corresponding control (Fig 1)

*Ribonuclease*—When a paramycin paste was incubated with varying concentrations of ribonuclease (RNase) in the presence of  $Mg^{++}$  ions (29),

no inactivation of paramecin, other than the inactivation normally occurring at the pH of 7.4, was found to occur (Table V). It is also clear from Table V that  $Mg^{++}$  ions do not affect the inactivation of paramecin.

*Desoxyribonuclease*—The most revealing experiments were those conducted with desoxyribonuclease. It can be seen from Fig. 2 that paramecin is inactivated rapidly when incubated with varying amounts of desoxyribonuclease.

TABLE V

*Incubation of Paramecin with Ribonuclease*

The ribonuclease and  $MgSO_4$  were dissolved in phosphate buffer, pH 7.4. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min	20 min	40 min	60 min
Control 1	4.0 cc paste	100.0	88.1	76.5	65.5
" 2	1.0 " buffer, pH 7.4				
	4.0 " paste	100.0	84.5	70.5	64.5
	0.5 " buffer, pH 7.4				
" 3	0.5 " $Mg^{++}$ solution, 0.25 M				
	4.0 " paste	100.0	85.6	73.4	65.0
	0.5 " buffer, pH 7.4				
	0.5 " RNase, 200 $\gamma$ per cc				
RNase, 20 $\gamma$ per cc	4.0 " paste	100.0	81.0	74.0	65.5
	0.5 " RNase, 200 $\gamma$ per cc				
	0.5 " $Mg^{++}$ solution, 0.25 M				
" 2 " " "	4.0 " paste	100.0	82.5	73.1	63.1
	0.5 " RNase, 20 $\gamma$ per cc				
	0.5 " $Mg^{++}$ solution, 0.25 M				
" 0.2 " " "	4.0 " paste	100.0	84.1	71.9	63.9
	0.5 " RNase, 2 $\gamma$ per cc				
	0.5 " $Mg^{++}$ solution, 0.25 M				

Desoxyribonuclease is specifically activated by  $Mg^{++}$  and  $Mn^{++}$  ions in concentrations as low as 0.003 M. The activation by  $Mg^{++}$  ions is inhibited by citrate in a concentration of 0.01 M, while the  $Mn^{++}$  activation is not (30). Experiments were designed to test whether the inactivation of paramecin by desoxyribonuclease was prevented by omitting magnesium from the substrate and whether addition of citrate to the complete test solution would have any influence on the inactivation of paramecin by desoxyribonuclease in the presence of either magnesium or manganese ions. It is clear from Fig. 3 that magnesium in a concentration

of 0.003 M is essential for the inactivation of paramycin by the enzyme. A slight inactivation is found if magnesium is omitted from the substrate. However, since no special precautions were taken to exclude all traces of magnesium from the solutions, this slight inactivation might be due to the

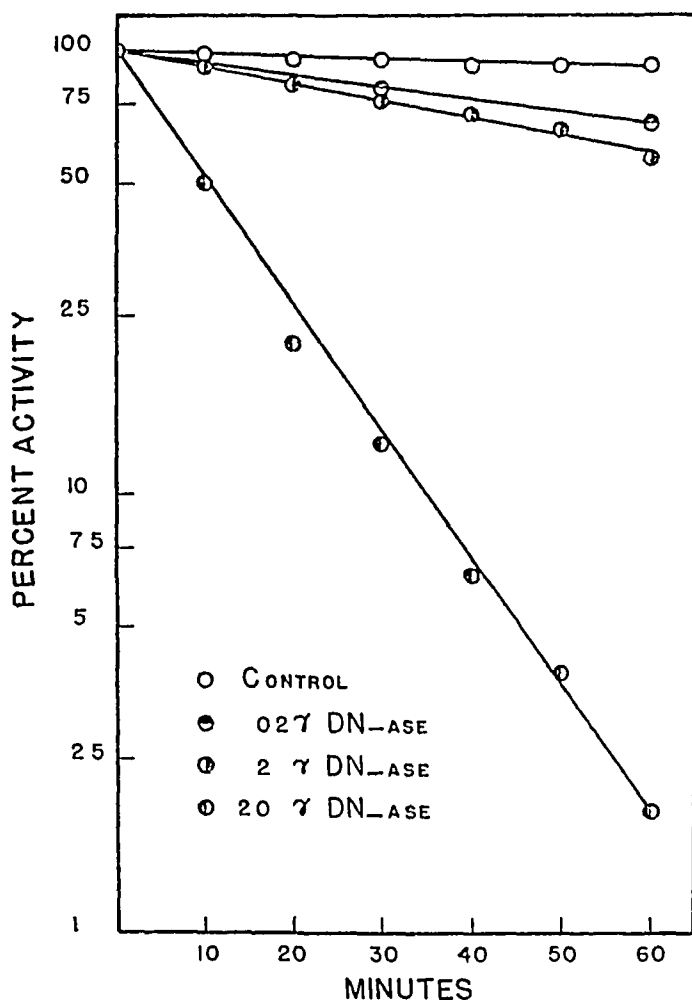


FIG. 2. Inactivation of paramycin by different concentrations of desoxyribonuclease. The paste of *Paramecium aurelia* was prepared by breaking the animals up in a 0.01 M phosphate buffer, pH 7.6, containing 0.25 per cent gelatin and 0.003  $Mg^{++}$  ions. The desoxyribonuclease was also dissolved in this buffer, in such a concentration that the final concentration in the test was like that indicated in the figure.

presence of traces of this metal in the substrate. The addition of citrate prevents paramycin breakdown when magnesium ions are the activators of desoxyribonuclease (Table VI). When manganese ions are present in the substrate, addition of citrate is ineffective (Table VII).

*Protein B*—The outcome of these experiments can be interpreted to mean

that desoxyribonucleic acid is an integral part of paramycin. However, Laskowski *et al* (12, 31) have reported the isolation of a proteolytic enzyme,

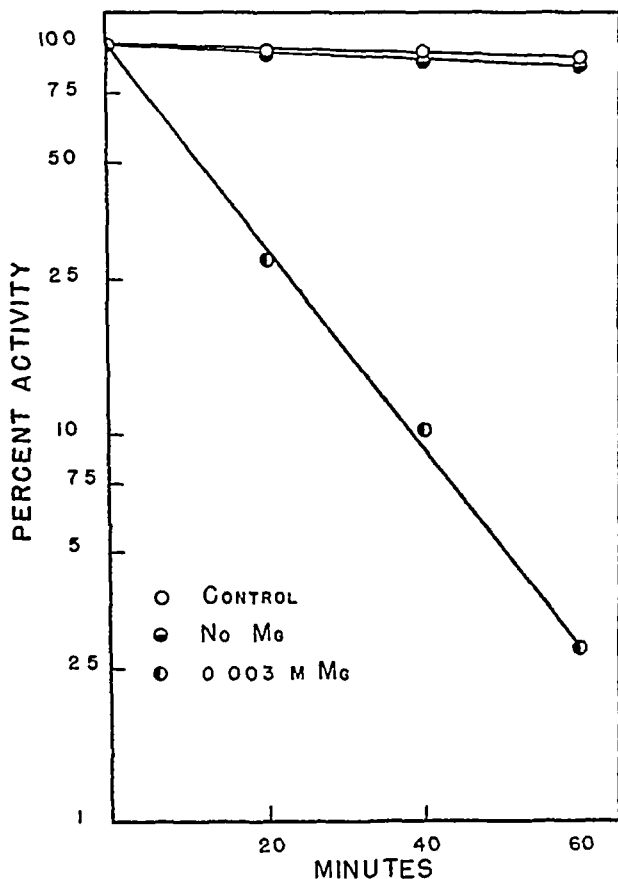


FIG 3 The influence of  $Mg^{++}$  ions on the inactivation of paramycin by desoxyribonuclease. The composition of the medium in the different runs was as follows: ○, 3.0 cc of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent), 1.0 cc of  $Mg^{++}$  solution, 0.015 M (pH 7.6, gelatin 0.25 per cent), 1.0 cc of buffer (pH 7.6, gelatin 0.25 per cent); ●, 3.0 cc of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent), 1.0 cc of desoxyribonuclease in phosphate buffer (pH 7.6, gelatin 0.25 per cent, DNase, 100  $\gamma$  per cc), 1.0 cc of buffer (pH 7.6, gelatin 0.25 per cent); ●, 3.0 cc of paste made in phosphate buffer (pH 7.6, gelatin 0.25 per cent), 1.0 cc of desoxyribonuclease (pH 7.6, gelatin 0.25 per cent, DNase, 100  $\gamma$  per cc), 1.0 cc of  $Mg^{++}$  solution, 0.015 M (pH 7.6, gelatin 0.25 per cent).

presumably of the chymotrypsin type, from desoxyribonuclease preparations, prepared according to the method of McCarty (30). It could there-

fore be possible that the observed inactivation of paramecin by desoxyribonuclease preparations was entirely due to contamination of desoxyribonuclease by a proteolytic enzyme. In order to test whether the observed inactivation of paramecin by desoxyribonuclease was indeed due to a specific action of the enzyme, the action of protein B, the proteolytic enzyme obtained from desoxyribonuclease preparations by Laskowski, was

TABLE VI

*Influence of  $Mg^{++}$  and Citrate on Inactivation of Paramecin by Desoxyribonuclease*

Desoxyribonuclease,  $MgSO_4$ , and Na citrate were dissolved in phosphate buffer, pH 7.6. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc. of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min	20 min	40 min	60 min
Control 1	3.0 cc paste	100.0	93.7	91.7	90.0
" 2	2.0 " buffer, pH 7.6				
	3.0 " paste	100.0	94.3	92.4	89.4
" 3	1.5 " buffer, pH 7.6				
	0.5 " $Mg^{++}$ solution, 0.03 M				
	3.0 " paste	100.0	92.5	92.0	88.8
" 4	1.5 " buffer, pH 7.6				
	0.5 " citrate, 0.1 M				
	3.0 " paste	100.0	96.0	92.0	91.0
DNase + $Mg^{++}$	1.0 " DNase, 100 $\gamma$ per cc				
	1.0 " buffer, pH 7.6				
	3.0 " paste	100.0	23.4	6.7	3.3
" + " + citrate	1.0 " DNase, 100 $\gamma$ per cc				
	0.5 " $Mg^{++}$ solution, 0.03 M				
	0.5 " buffer, pH 7.6				
	3.0 " paste	100.0	94.8	91.8	87.5
	1.0 " DNase, 100 $\gamma$ per cc				
	0.5 " $Mg^{++}$ solution, 0.03 M				
	0.5 " citrate, 0.1 M				

tested. It is evident from Table VIII that this enzyme, even when tested in concentrations 1000 times greater than those used in the desoxyribonuclease experiments, does not inactivate paramecin.

#### DISCUSSION

It can be tentatively concluded from the experiments reported above that paramecin is a desoxyribonucleoprotein. Inactivation of the killer principle by pepsin and chymotrypsin indicates that a protein is an integral part of the paramecin essential for its activity. The specific inactivation by

TABLE VII

*Influence of Mn<sup>++</sup> and Citrate on Inactivation of Paramycin by Desoxyribonuclease*

Desoxyribonuclease, MnSO<sub>4</sub>, and Na citrate were dissolved in phosphate buffer, pH 7.6. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Composition of medium	Per cent activity			
		0 min	20 min	40 min	60 min
Control 1	3.0 cc paste	100.0	97.5	91.0	91.0
" 2	2.0 " buffer, pH 7.6				
	3.0 " paste	100.0	95.6	93.7	91.7
	1.5 " buffer, pH 7.6				
" 3	0.5 " Mn <sup>++</sup> solution, 0.03 M				
	3.0 " paste	100.0	94.5	92.5	90.8
	1.5 " buffer, pH 7.6				
" 4	0.5 " citrate, 0.1 M				
	3.0 " paste	100.0	94.2	94.2	92.0
	1.0 " DNase, 100 $\gamma$ per cc				
	1.0 " buffer, pH 7.6				
DNase + Mn <sup>++</sup>	3.0 " paste	100.0	31.0	5.7	3.2
	1.0 " DNase, 100 $\gamma$ per cc				
	0.5 " Mn <sup>++</sup> solution, 0.03 M				
	0.5 " buffer, pH 7.6				
" + " +	3.0 " paste	100.0	31.6	6.1	2.5
citrate	1.0 " DNase, 100 $\gamma$ per cc				
	0.5 " Mn <sup>++</sup> solution, 0.03 M				
	0.5 " citrate, 0.1 M				

TABLE VIII

*Action of Protein B on Crude Extracts of Paramycin*

Protein B was dissolved in phosphate buffer, pH 7.8. Samples were taken at the specified time intervals and diluted 1:500 before testing. 1 cc of the dilution was distributed over ten depressions containing sensitive *Paramecium aurelia*. Dead and affected animals were counted after 48 hours.

	Per cent activity			
	0 min	20 min	40 min	60 min.
Control, pH 7.8	100.0	98.0	92.0	92.0
Protein B, 20 mg per cc	100.0	97.2	94.5	91.0
" " 10 " " "	100.0	97.0	88.5	88.5
" " 1 " " "	100.0	90.7	87.5	85.5

desoxyribonuclease suggests that a desoxyribonucleic acid is also required for the activity. It is fully realized that a definite conclusion as to the



chemical nature of paramecin can be reached only after this compound has been isolated in the pure and active state

Nevertheless, several important facts emerge from this study. The inactivation of paramecin by both pepsin and chymotrypsin and the fact that neither papain nor trypsin influences the activity of paramecin (the apparent inactivation of paramecin by trypsin is not due to proteolytic action of this enzyme) indicate that basic amino acids such as arginine and lysine are probably not essential for the activity of paramecin, for both pepsin and chymotrypsin require as groups in the side chain of the substrate phenylalanine or tyrosine residues, while the requisite groups for trypsin are those of the arginine and lysine residues (32)

The available evidence seems to indicate the presence of a desoxyribonucleoprotein in the cytoplasm of *Paramoecium aurelia*. Bensley (33), Hoeft (34), and Lazarow (35) reported the isolation of a cytoplasmic component, plasmosin, of the desoxyribonucleoprotein type. Their view-point is not shared by Mirsky and Pollister (36), who maintain that plasmosin is located largely, if not entirely, in the nucleus of the cell, it being a constituent of chromatin. It seems to be generally agreed that nucleic acids of the desoxyribose type are never regular constituents of the cytoplasm, and any desoxyribonucleic acid found in cytoplasmic preparations is thought to be present there because the methods used for the isolation tend to dissolve the content of the nucleus, thereby liberating desoxyribonucleic acid of nuclear origin (37, 38). However, Sparrow and Hammond (39) published evidence, based on the Feulgen reaction and on ultraviolet absorption data, that bodies containing desoxyribonucleic acid are present in the cytoplasm of meiotic stages of micro sporocytes from eight genera of plants. Furthermore, the presence of very small quantities of desoxyribonucleic acid in the cytoplasm has never been completely excluded (36-38, 40). It then would not seem unlikely that, in view of the extreme biological activity of paramecin, only minute traces of this compound were present (41).

The importance of desoxyribonucleic acid as a cellular determinant is well established through the work of Avery *et al* and McCarty (42-44) on the transforming principle of *Pneumococcus*. Amounts as small as 0.001  $\gamma$  will transform non-encapsulated R variants of *Pneumococci* into encapsulated S variants. Boivin and associates (45-47) have presented evidence that a desoxyribonucleic acid acts as a transforming principle in *Escherichia coli*, similarly changing the antigenic type. In *Paramoecium aurelia* a desoxyribonucleic acid is an integral part of a substance responsible for a specific killing action. Pieer (48) recently reported that Feulgen-positive bodies, having the physiological properties of the cytoplasmic factor  $\lambda$ , are in the cytoplasm of killer animals. Sonneborn (49) has presented

evidence that sensitive animals, homozygous for the gene K, can be transformed into true killer animals by exposure to large concentrations of cytoplasmic material obtained from killer animals. A suggestive working hypothesis could postulate that paramecin is closely related to the cytoplasmic factor which then in *Paramecium aurelia* would have a similar function as the transforming principle of *Pneumococcus* and *Escherichia coli*. The apparent difference between the two cases is that transformation in *Paramecium aurelia* is brought about by a desoxyribonucleoprotein, while desoxyribonucleic acid alone is capable to act as a transforming agent for *Pneumococcus* and *E. coli*. However, it might be possible that in the latter case desoxyribonucleic acid acts as a prosthetic group of the transforming principle, *Pneumococcus* and *E. coli* being able to synthesize the protein moiety. *Paramecium aurelia*, unable to synthesize the protein part, would require the whole unsplit desoxyribonucleoprotein.

#### SUMMARY

Paramecin is inactivated by pepsin, chymotrypsin, and desoxyribonuclease, indicating the presence of a protein and a desoxyribonucleic acid in the compound. Both parts are essential for the killing action of paramecin. The inactivation of paramecin by desoxyribonuclease is specifically activated by magnesium and manganese ions. The inactivation of paramecin by desoxyribonuclease in the presence of magnesium ions is inhibited by citrate, in contrast to the inactivation by this enzyme in the presence of manganese ions, which is unaffected by the addition of citrate to the substrate. The inactivation by desoxyribonuclease is not due to the presence of a contaminating proteolytic enzyme. This enzyme, protein B, does not inactivate paramecin in concentrations 1000 times greater than that of desoxyribonuclease. Lysozyme, hyaluronidase, papain, and ribonuclease did not inactivate paramecin. Trypsin apparently formed an inactive complex with paramecin, which dissociated on dilution. The implications of these findings in relation to the cytoplasmic factor are discussed.

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# THE PREPARATION AND PROPERTIES OF A LYSOPHOSPHOLIPASE FROM *PENICILLIUM NOTATUM*\*

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In a previous communication (1) the name *phospholipase* was used to describe the enzyme in various venoms which converts lecithins and cephalins to lysolecithins and lysocephalins, with liberation of unsaturated fatty acids. The present work describes a powerful enzyme occurring in *Penicillium notatum*, which acts upon lysophospholipides to produce glycerylphosphorylcholine (or glycerylphosphorylethanolamine), with liberation of saturated fatty acids. This enzyme will be called *lysophospholipase*. Phospholipase and lysophospholipase are terms synonymous with lecithinase A and lecithinase B (2), respectively.

Investigation of the enzymatic degradation of the phospholipides has met with scant success when animal tissues have served as sources of the various enzymes concerned. This has not been due to absence of such enzymes from the tissues, for their existence has been amply proved through the study of autolytic processes (3, 4). In order to improve our understanding of the phospholipide-hydrolyzing enzymes, it has been considered advisable to look for sources of the individual enzymes which would make possible the study of each step in the degradation process. The classic example among such sources is, of course, the extremely active phospholipase of venoms, previously mentioned. In 1933 Contardi and Ercoli (5) described a lysophospholipase obtained from rice bran, rice embryos, and *Aspergillus oryzae*, whose activity was, however, quite low. *Penicillium notatum* has now been found to be an excellent source of this enzyme. Methods have been devised for the quantitative assessment of the reaction and for the determination of lysophospholipase activity. Certain properties of the enzyme have also been examined. The investigation has been discontinued.

## EXPERIMENTAL

*Enzyme*—*Penicillium notatum*<sup>1</sup> was surface-cultured in 500 ml Erlenmeyer flasks, on a corn steep, lactose-containing medium (6), for 5 to 8 days.

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<sup>1</sup> The *Penicillium* strain used was kindly supplied by Dr. G. B. Reed.

at 25° The mat of abundantly sporulating mold was then removed from the culture medium, washed with water, and dried superficially by pressing gently between filter papers After crumbling the mat between the fingers, drying was completed *in vacuo* over anhydrous calcium sulfate (Drierite) Each culture yielded more than 1 gm of dry mold, which was stored at 4° until required

Extracts of this material were prepared by pulverizing 5 gm portions, mixed with a little sand, adding 50 ml of distilled water plus 5 drops of toluene, and autolyzing for 24 hours at 20–25° After filtration and washing with suction, the combined filtrates (60 ml) were dialyzed for 24 hours at 4°, with inside stirring, against 20 liters of running distilled water After dialysis, one of two procedures was followed (1) The extract was diluted to 75 ml and stored at 4° under toluene, (2) the solution, in the dialysis sac, was suspended in glycerol and concentrated to about 14 ml, after which it was removed from the sac, diluted to 75 ml with glycerol and stored at 4°

Aqueous extracts lost about 80 per cent of their activity when stored at 4° for 2½ months During the same period of time glycerol preparations remained fully active<sup>2</sup> The nitrogen content of the dialyzed aqueous extracts varied from 0.280 to 0.380 mg per ml

*Substrate*—Lysophospholipides were prepared from egg yolks by a simplification of King's procedure (7), in which troublesome concentration of extracts *in vacuo* was eliminated After formation of the lysophospholipides by incubation of 60 yolks with moccasin venom (*Aghkistrodon piscivorus*), the reaction mixture (3 liters) was extracted with acetone (6 liters) at 50°, and filtered rapidly while still hot The residue was reextracted twice with acetone (2 liters) and the combined filtrates cooled to 20° To this were then added 90 ml of saturated aqueous cadmium chloride solution, and the mixture was cooled to 0° The copious precipitate was filtered off rapidly and extracted 5 times in a Waring blender with 300 ml portions of cold acetone It was then dried *in vacuo*, following which lysophospholipides were isolated from the cadmium chloride complex by the usual methods Yield, 35 gm, P 5.70 per cent, N 2.72 per cent, NH<sub>2</sub>-N (8) 0.552 per cent, iodine value of constituent fat acids 4

Lysophospholipide sols were prepared as required, usually at concentrations of 1 or 0.5 per cent, by shaking with water or buffer solution at 40–50° Such sols were stable at 30°, but precipitated slightly at 25° and heavily at 4° They were chemically stable, at pH 4.0, for a period of several weeks and after storage at 4° were readily reformed by gentle heating

<sup>2</sup> As a result of the considerable stability of lysophospholipase in solution, extensive efforts to purify the enzyme, or to produce dry preparations, were not made It could be precipitated partially from solution by saturated ammonium sulfate, by acetone, and by acid (pH 3.0), but on the basis of nitrogen content, none of these treatments increased the activity appreciably

*Reaction Mixture*—Unless otherwise specified, the reaction mixture contained 1 ml of 1 per cent lysophospholipides and 0.04 ml of enzyme, in a total volume of 2.2 ml. All components of the final mixture were prepared in veronal-acetate buffer, pH 4.0. For the reaction, all components except enzyme were mixed in glass-stoppered test-tubes, and equilibrated for 20 minutes in a water bath maintained at 30°. The enzyme was added, and after 5 minutes a 1 ml aliquot was removed into precipitation tubes as described below.

Under these conditions, the originally clear solution became quite turbid, owing to liberation of insoluble fatty acids, and if allowed to stand, formed a stiff gel. Degradation of the lysophospholipides varied from 15 to 50 per cent after 5 minutes, depending upon the age of the lysophospholipase used.

*Quantitative Determination of Reaction*—Preliminary experiments indicated that nephelometric or viscosimetric methods could not be applied as quantitative assessments of the reaction. Efforts were made, therefore, to separate the substrate from one or both of the reaction products. It was found that a satisfactorily complete separation of lysophospholipides and fatty acids from glycerylphosphorylcholine<sup>3</sup> could be made by the colloidal iron-magnesium sulfate method of Folch and Van Slyke (9). Lysophospholipides in the precipitate were then determined by analysis for phosphorus.

Details of the procedure were as follows. 1 ml aliquots of the reaction mixture were added to 12 ml conical centrifuge tubes containing 2.8 ml of veronal-acetate buffer, pH 9.2, plus 4.2 ml of water, and stirred immediately. The alkaline pH of this mixture effectively stopped the reaction. To the tubes were then added with stirring 0.5 ml of colloidal iron ( $\text{Fe}_2\text{O}_3$ , 5 per cent dialyzed) and 0.5 ml of half saturated magnesium sulfate solution. Addition of these acid reagents lowered the pH of the mixture to about 6.5. The tubes were centrifuged for 3 minutes, the supernatant solution containing the GPC, decanted, and the precipitates washed with 10 ml of 0.05 saturated magnesium sulfate solution. To the centrifuged, washed precipitates were then added 3 ml of water, in which the precipitates were suspended by thorough stirring, and transferred by aspiration to micro-Kjeldahl digestion flasks. The centrifuge tubes were washed carefully with two 1 ml portions of sulfuric acid (86 per cent by volume), and the washings transferred to the digestion flasks. Digestion was carried out by adding 0.200 gm of potassium sulfate-copper sulfate mixture (9:1) and boiling for 1 hour. An acid-washed Hengar granule effectively overcame the tendency to bump. After digestion, any precipitate present was

<sup>3</sup> The abbreviation GPC will be used to denote the mixed esters, glycerylphosphorylcholine and glycerylphosphorylethanolamine.

filtered off, and phosphorus was determined colorimetrically (10) The presence of iron, magnesium, copper, and potassium ions was without effect on the determination

Identical amounts of lysophospholipides carried through the entire precipitation and digestion procedures showed a maximum variation in the analyses of  $\pm 1$  per cent The absolute amounts of phosphorus recovered in the precipitate, however, varied from 97 to 98 per cent of those found by direct analysis of lysophospholipides There was, thus, a constant error of 3 per cent in the precipitation method No correction was made for this loss

In order to test the completeness with which lysophospholipide phosphorus and GPC phosphorus could be separated, varying amounts of the

TABLE I  
*Separation of Lysophospholipide Phosphorus from GPC Phosphorus*

P found*	GPC P added	GPC P as per cent of total P	Recovery of lysophospholipide P
mg	mg		per cent
0.270	0		97.5
0.272	0.0106	3.7	98.2
0.272	0.0212	7.1	98.2
0.270	0.0530	16.0	97.5
0.270	0.106	27.7	97.5
0.270	0.212	43.4	97.5

\* Lysophospholipide P by direct analysis (without precipitation by iron-magnesium mixture) = 0.277 mg All values are averages of duplicate samples

latter<sup>4</sup> were added to a constant amount of lysophospholipides, following which the precipitation procedure was applied As shown in Table I, the separation was complete

The extent of the enzymatic reaction was calculated by comparison with the precipitated lysophospholipide phosphorus from a control tube Since, in experimental tubes, the enzyme itself contained a small amount of precipitable phosphorus, an appropriate correction was made

Under the conditions prescribed, addition of iron-magnesium to the precipitation tubes containing alkaline reaction mixture could be delayed 15 minutes, and after this addition the tubes could stand for at least 60 minutes before transfer of their contents to digestion flasks In this way, it was possible to make twenty or more determinations in a single experiment

Inorganic phosphate, if present, appeared in the precipitate with lyso-

<sup>4</sup> A sample of pure racemic  $\alpha$ -glycerylphosphorylcholine was kindly supplied by Dr. Erich Baer

phospholipides Glycerophosphate was partially precipitated Thus, the method was valid only in the virtual absence of enzymes liberating choline or inorganic phosphate, or both, from GPC It was possible to show that the enzyme preparation used liberated appreciable amounts of inorganic phosphate in the reaction mixture, at pH 4.0, only when present in high concentration (10 to 100 times the concentration ordinarily used) and over a prolonged time (4 to 8 hours) Similarly, in mixtures containing 5 times

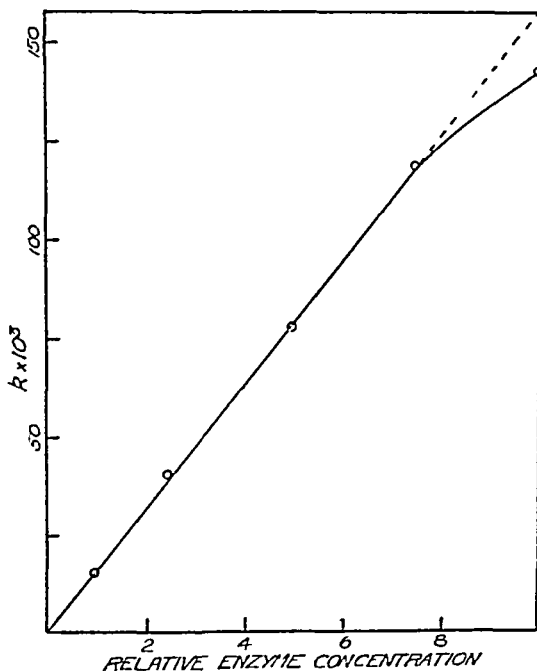


FIG 1 Direct proportionality between first order constants ( $k$ ) and lysophospholipase concentration, during the initial stages of reaction The first point on the curve represents 7 per cent hydrolysis, the last point 53 per cent Reaction time, 5 minutes

the usual concentration of enzyme, incubated for 60 minutes, no free choline was determinable by the sensitive reneckate method Under the conditions specified, therefore, the only reaction occurring to a significant extent was the conversion of lysophospholipides to GPC and fatty acids

*Measurement of Activity*—During the initial stages of the reaction the decrease in lysophospholipide phosphorus, in a given time, was not directly proportional to enzyme concentration Under certain conditions, however, a first order reaction was simulated, the appropriate constant ( $k$ ) being proportional to enzyme concentration over a considerable range (Fig 1)



These conditions were (1) a reaction temperature of 30° (2) a reaction time of 15 minutes or less, (3) a per cent reaction less than 10

*Substrate Specificity*—Repeated attempts were made to secure a reaction between lysophospholipase and lecithins or cephalins, all of them unsuccessful. The following phospholipide preparations were used: egg yolk and brain<sup>5</sup> lecithins purified according to Pangborn (11), petroleum ether-soluble egg yolk phospholipides (lecithins and cephalins), and the acetone-insoluble fraction of a commercial soy bean lecithin. With quantities of enzyme which extensively decomposed lysophospholipides (60 to 70 per cent) within 10 minutes, no action on lecithins was observed even after 1 hour.

Since the lysophospholipides in routine use as substrate for the enzyme contained 20.3 per cent of their total nitrogen as amino nitrogen, it was of interest to discover whether lysocephalins were reactive. In the absence of a method for obtaining pure lysocephalins, an indirect approach to the problem was made. 192 mg. of lysophospholipides (containing 1.11 mg. of amino nitrogen) were incubated for 1 hour with excess enzyme, at which time the reaction was 84 per cent complete. After separation of the residual lysophospholipides by the usual method, the GPC-containing solution was dried *in vacuo*, and analyzed for ethanolamine nitrogen (12). 0.573 mg. of ethanolamine nitrogen was found, corresponding to 52 per cent of the total amino nitrogen available for reaction. Lysophospholipase, therefore, acted upon lysocephalins as well as upon lysolecithins. Serine nitrogen (12) was not present in the substrate, and the enzyme itself contained neither ethanolamine nor serine nitrogen.

*pH Optimum*—The activity of lysophospholipase was determined at hydrogen ion concentrations varying from pH 2.88 to 6.43. The veronal-acetate buffer used exhibits constant ionic strength over this range (13). Sufficient enzyme was added to produce 31 per cent reaction at the most favorable hydrogen ion concentration. From the results (Fig. 2) it is seen that the optimum acidity for the reaction was rather sharply defined in the pH range 3.8 to 4.4. Contardi and Ercoli (5) found an optimum at pH 3.5 for the corresponding enzyme from *Aspergillus oryzae*.

*Heat Stability*—Fig. 3 contains a group of curves illustrating the resistance of lysophospholipase to various temperatures and hydrogen ion concentrations. In establishing these curves, solutions containing 0.6 ml. of enzyme extract per ml., at varying hydrogen ion concentrations, were heated at a given temperature (41°, 50°, or 61°) for a given time (15 or 30 minutes). The solutions were then cooled quickly, and then activity measured and compared with untreated enzyme. It is evident from the figure that the lysophospholipase exhibited a maximum heat stability near pH 4.5 and

<sup>5</sup> The brain lecithin was a preparation kindly made available by Dr. R. G. Sinclair.

was rapidly inactivated at a pH greater than 7.0, at temperatures of 41° or higher

*Activation and Inhibition*—In general, lysophospholipase was resistant to most of the common enzyme inhibitors, and no activator was discovered

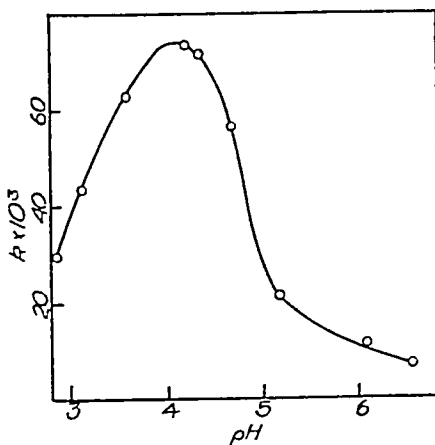


FIG 2 pH-activity curve for lysophospholipase

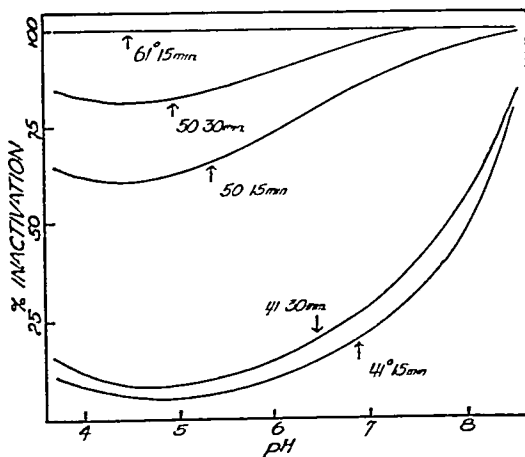


FIG 3 Stability of lysophospholipase to heat and hydrogen ion concentration  
Per cent inactivation =  $100(1 - (A_{\text{treated}}/A_{\text{control}}))$

Cyanide was the most effective inhibitor among those tested (Table II), a 0.01 M concentration suppressing the reaction almost completely. Silver and copper ions were less effective. Calcium, magnesium, and cobalt ions did not inhibit the reaction, nor did sodium azide, cysteine, hydrogen sulfide, or hydrogen peroxide. The enzyme solution was slowly inactivated, pre-

sumably by surface denaturation, when air or nitrogen was bubbled through it

As products of the reaction, GPC and fatty acids might be considered as inhibitors, since the reaction in the presence of the usual concentration of enzyme proceeded only to 60 to 70 per cent completion. Palmitic and linoleic acids (the latter a liquid acid not actually occurring in the substrate), added to the reaction mixture in amounts approximating their maximum concentration in the substrate, slightly inhibited the reaction. GPC, on the other hand, exerted no effect. Quite possibly the reaction was effectively suppressed before a chemical equilibrium was reached, as a result of

TABLE II  
*Inhibition of Lysophospholipase\**

Inhibitor	Concentration	Inhibition	Inhibitor	Concentration	Inhibition
	<i>mole per l</i>	<i>per cent</i>		<i>mole per l</i>	<i>per cent</i>
Palmitic acid	0.0094	18	AgNO <sub>3</sub>	0.01	48
	0.00094	4		0.002	13
Linoleic acid	0.0089	11		0.001	8
	0.00089	6	KCN	0.0001	4
GPC	0.0097	2		0.01	97
	0.00097	0		0.001	87
CuSO <sub>4</sub>	0.05	51		0.0002	48
	0.005	36		0.0001	12
	0.0001	21		0.00002	0

\* Copper, silver, and cyanide ions were incubated with lysophospholipase for 30 minutes at 30° before addition to the substrate. During this time their concentration was 11 times that indicated in the table.

the gel formation which occurred after some 60 per cent of the lysophospholipides were decomposed.

Francioli (14) reported the complete inhibition by physostigmine chloride of a comparable enzyme found in wasp venom. In our experiments physostigmine sulfate did not inhibit in a concentration as high as 1.0 mg. per ml., which was some 50 times greater than the concentration employed by Francioli. Since neither physostigmine chloride nor wasp venom was available to us for direct test, no explanation of this discrepancy in results can be advanced.

#### DISCUSSION

The name lysophospholipase implies a certain specificity of the enzyme for lysophospholipides. Contardi and Ercoli (5) and Francioli (14), after examining the corresponding enzyme derived from rice bran and embryos,

*Aspergillus*, and wasp venom, concluded that phospholipides also served as substrate. The former workers, however, did not demonstrate in their preparations the absence of a true phospholipase. The results of Francioli, on the other hand, were based upon evidence acquired through the use of physostigmine as a lysophospholipase inhibitor. Our preparations, which were many times more active than those hitherto described, were completely inactive with respect to lecithins and cephalins, nor was it possible to demonstrate any inhibition of the reaction by physostigmine. Lysophospholipase from *Penicillium* may be regarded, therefore, as being specific for lysophospholipides.

Failure of *Penicillium* extracts to liberate inorganic phosphate from lysophospholipides or GPC was due not to lack of the acid phosphatase abundantly present in molds, but to a deficiency of GPCase, without which glycerophosphate could not be formed. Extracts of *Aspergillus oryzae*, prepared in the laboratory or obtained commercially,<sup>6</sup> contained considerable GPCase.

The lysophospholipase preparation used was relatively pure, i.e., free of dialyzable substances, and low in nitrogen content. Each reaction tube, as ordinarily prepared, contained only 0.010 to 0.016 mg of nitrogen added as enzyme. Efforts directed towards further purification of lysophospholipase should be rewarding.

#### SUMMARY

Lysophospholipase, a highly active enzyme specific in its action for lysophospholipides, was prepared from *Penicillium notatum*. The products of the reaction were saturated fatty acids and glycerylphosphorylcholine or glycerylphosphorylethanolamine (GPC).

Quantitative methods for determining the extent of the reaction were based upon the separation of residual lysophospholipides and liberated fatty acids from GPC by precipitation of the former with colloidal iron-magnesium sulfate mixture.

Lysophospholipase activity was determined, under prescribed conditions, by evaluation of the first order reaction constant.

The enzyme was readily inactivated by heat at a slightly alkaline reaction, by cyanide, and, less readily, by heavy metal ions. No activator was discovered. Optimum activity was at pH 4.0.

Grateful acknowledgment is made to Miss Katherine Justus for many of the analyses, and to Miss Eve Minovitch for the determinations of ethanolamine and serine nitrogen.

<sup>6</sup> By courtesy of Mr. F. F. Taylor, Takamine Laboratory, Inc., Clifton, New Jersey.

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# DETERMINATION OF PROTEIN-BOUND IODINE\*

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The growing interest in chemical evaluation of thyroid function by use of the plasma level of protein-bound iodine (PI) is attested by the increasing numbers of reports in which this criterion is employed, both chemically and experimentally. However, the specific techniques available for such a determination do not yet seem sufficiently reliable. The purpose of this communication is to describe a procedure evolved in this laboratory which is consistent and sensitive in our hands.

The complete determination of plasma PI involves four distinct steps: (1) precipitation and washing of the plasma proteins, (2) digestion of the protein, leaving the PI in inorganic form, (3) distillation of the inorganic iodide, (4) actual determination of the inorganic iodide. The shortened technique of Salter (4), in which a dry ashing with  $\text{NaOH}$  and  $\text{Na}_2\text{CO}_3$  was used to eliminate the need for distillation, has not proved satisfactory in our experience, and no other short cuts have been proposed.

In 1940, Chaney (1) described an all-glass still for use with a chromic-sulfuric acid digestion mixture, iodide catalysis of the reduction of ceric ions by arsenious acid was employed for the ultimate colorimetric determination of iodine. Although other workers have adopted the Chaney still (7), the highly sensitive ceric-arsenious catalysis method was generally avoided until Taurig and Chaikoff reported an entire procedure for plasma iodine (8). The iodide catalysis reaction had been extensively studied in 1937 by Sandell and Kolthoff (5), who demonstrated a marked enhancement of the iodide effect by the presence of a relatively high concentration of chloride. We have found that the use of chloride in optimum concentration improves the sensitivity 30 to 80 per cent with no sacrifice in reproducibility. None of the recently reported techniques has taken advantage of this appreciable increase in sensitivity.

## EXPERIMENTAL

### *Reagents—*

*Distilled water* Although in the past it has been widely reported necessary to take the extraordinary precautions of freshly distilling the water from alkali, we have found it quite satisfactory merely to redistil once

\* Aided by a grant from the United States Public Health Service

distilled water in an all-glass still. Since this is a low iodine region, the deficiency of iodine in the water may facilitate this. Furthermore, water so prepared and stored in a stoppered Pyrex flask for as long as 2 months at summer temperatures did not show any change from its extremely low iodine content or any effect on the recovery of iodide quantitatively added to it. All other reagents are made up in the double distilled water.

*Somogyi precipitating reagents (6)* The acid zinc sulfate solution contains 12.5 gm of  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  and 125 ml of 0.25 N  $\text{H}_2\text{SO}_4$  per liter. NaOH is made up to 30 gm per liter, and the two solutions are balanced until 50 ml of the acid zinc sulfate require between 6.7 and 6.8 ml of the alkali to show a permanent pink to phenolphthalein. A blank must be run on these reagents, carried through the washing procedures to be described.

*Sulfuric acid solutions* 70 per cent by weight. 780 ml of concentrated sulfuric acid, special, As- and N-free, are slowly added, with cooling, to 600 ml of water in a 2 liter Pyrex flask. Thorough mixing should then be carried out.

1.0 N. 28 ml of concentrated acid are carefully added to 900 ml of water, and the solution cooled and made to 1 liter.

*Chromic oxide* 600 gm are dissolved in water, and made to 1 liter. This material has often been found to be contaminated, we have tested several batches of technical grade  $\text{CrO}_3$  and found some to be very low in iodine. These particular samples are far less expensive than the high purity material, which is equally apt to be contaminated.

*Sodium sulfite* A 10 per cent solution is used, which is made up fresh for each series of distillations by dissolving 2 gm in 20 ml of water.

*Phosphorous acid, 50 per cent* 250 gm are dissolved in about 200 ml of water and made to 500 ml. If necessary the solution can be freed of iodine by boiling for  $\frac{1}{2}$  hour (with frequent addition of water), when cool, it is made to 500 ml. We have used the reagent from Fisher without boiling.

*Arsenious acid* 3.71 gm of  $\text{As}_2\text{O}_3$  are dissolved in 50 ml of N NaOH with stirring. 200 ml of water are added and the solution neutralized with  $\text{H}_2\text{SO}_4$  (requiring about 2.5 ml of the 70 per cent solution). Then 54 ml of the 70 per cent  $\text{H}_2\text{SO}_4$  are added, and the solution made to 500 ml. 3.125 gm of iodide-free NaCl are dissolved in the 500 ml of reagent to avoid the need of another solution.

*Ceric sulfate* 12 gm of ceric ammonium sulfate (G. Frederick Smith) are stirred into 500 ml of 3.5 N  $\text{H}_2\text{SO}_4$ . This will be turbid at first, clearing up within  $\frac{1}{2}$  hour upon occasional stirring.

*Iodide standards* Pure NaI is carefully dried in a desiccator, 118.1 mg are then dissolved and diluted to 1 liter. This stock solution contains 100  $\gamma$  of I per ml, and must be appropriately diluted to yield the 0.005 to 0.100  $\gamma$  of I desired as standards.

*Special Apparatus*—The Riggs modification of the Chaney still was employed as described by Talbot *et al* ((7) p 481) except that a 250 ml flask was substituted for the 500 ml flask. All grease was cleaned from both stop-cocks, and water was used as the only lubricant. Care must be taken to prevent freezing of the stop-cocks due to drying.

### *Procedure*

This section falls logically into the four categories mentioned earlier.

1 *Protein Precipitation and Washing*—2 ml of ovalated or heparinized plasma are precipitated by Somogyi's zinc sulfate reagent in a 50 ml round bottom centrifuge tube. After a 10 minute period of centrifuging, the supernatant is poured off, and the precipitate washed free of inorganic iodide by four successive washings each with 25 ml of iodine-free distilled water. After the last washing and centrifuging, the protein is dissolved in 5 ml of 70 per cent  $\text{H}_2\text{SO}_4$  and transferred to the digestion flask. Four further 5 ml portions of acid and one final 5 ml portion of water are employed to insure complete transfer.

2 *Digestion*—3 ml of 60 per cent  $\text{CrO}_3$  are added, plus a few glass beads, carborundum particles, or other antibumping agent. The digestion is carried out over a small flame until sulfuric acid fumes appear. The flask is allowed to cool, 15 ml of water are added, and the digestion repeated.

3 *Distillation*—25 ml of water and a few fresh beads are added and the chromic acid crystals dissolved by rotation just before distilling in order to make use of the heat generated by dilution. The flask is attached to the still and a micro burner flame placed beneath. Enough water is added through the upper opening of the trap to fill the region of attachment of the stop-cock, and then 0.5 ml of a 10 per cent  $\text{Na}_2\text{SO}_3$  solution is allowed to drain down the walls of the bulge above the trap. Most of this will collect in the low portion of the trap, and soon after the distillation of water vapor has begun, condensation results in the trap being completely closed with fluid.

The water-cooled condenser is connected and 5 ml of 50 per cent  $\text{H}_3\text{PO}_3$  are placed in the dropping funnel after it has been inserted into the free opening in the flask. All ground glass joints are lubricated with water before being assembled. After boiling has continued until water vapor has entered the condenser and has started to drip into the return tube, the  $\text{H}_3\text{PO}_3$  is slowly blown into the flask by gentle pressure. The distillation is continued for 10 minutes after reduction is completed. To terminate the distillation, the flame is turned off and the trap is immediately drained into a 22 × 175 mm test-tube calibrated at 25 ml. The condenser is raised clear and the walls of the trap rinsed down with five successive 2.5 ml quantities of water, each washing being added to the distillate in the test-tube.



1 ml of N  $\text{H}_2\text{SO}_4$  is added to each combined distillate and washings. The resulting sulfurous acid is decomposed and  $\text{SO}_2$  blown off by aeration at about 25 liters per minute while the tube is in boiling water. The tube is then cooled, the volume made to the 25 ml mark, and the solution thoroughly mixed.

4 *Colorimetric Determination of Iodide*—5 ml aliquots are pipetted from the 25 ml total volume into Klett-Summerson colorimeter tubes. 0.4 ml of arsenious acid is added, and the tubes are placed in a water bath accurately regulated to  $37^\circ$ . Two tubes containing 5.0 ml of water and 0.4 ml of arsenious acid should be routinely used for blank determinations in each series of twenty. Ceric ammonium sulfate is next added, but this must be done on a definite time schedule, since only one measurement is to be made of a rate of reaction. Incubation for the ceric sulfate-arsenious acid reaction is carried out for 15 minutes, and  $\frac{1}{3}$  of a minute is allowed for each tube to be read in the colorimeter. At zero time, 0.5 ml of the ceric ammonium sulfate solution is added to the first tube, the contents are quickly mixed, and the tube replaced in the water bath. A 45 second interval is allowed, and then the procedure is repeated for the rest of the tubes. Thus, a maximum of twenty individual tubes or ten duplicate determinations can be handled in one series. 15 minutes after addition of the ceric solution to the first tube, it is removed from the bath, the outside quickly wiped clean and dry, and a reading obtained in the photoelectric colorimeter with No. 42 blue filter. The same 45 second interval should be ample for making each reading.

Because of the necessity for rigid adherence to a time schedule, performance must be checked at the start by including standards in each series of determinations. Once the routine has been thoroughly established, it is adequate to run only one set of standards, ranging from 0.01 to 0.10  $\gamma$ , as part of one series each time several sets of determinations are being performed.

The standard curve can be considered straight over only a restricted portion, and iodine values are best judged from an actual plot of reference values (Fig. 1). Blank values should be established for each new batch of reagents, and should be repeated occasionally as a check on contamination. After the blank has been deducted, calculations from these values should include the factor of 5 to cover the aliquot of 5 ml out of the 25 ml total volume and a factor of 50 to express the plasma PI in terms of micrograms per 100 ml of plasma.

#### *Comments and Precautions*

1 *Protein Precipitation and Washing*—Somogyi's zinc precipitation procedure as described has been found by far the most convenient. Heat

coagulation in a weakly acid medium is satisfactory, but requires constant attention. Trichloroacetic acid has been found unsatisfactory, at least partly because of the large amount of additional organic matter requiring digestion.

The precipitation technique has been applied successfully to tissues other than plasma by using the Potter homogenizer (3). 500 mg of liver, kidney, heart, or skeletal muscle are homogenized in 8 ml of the acid zinc sulfate reagent. This is poured into a 50 ml centrifuge tube, followed by four rinsings of the grinder, each with 4 ml of the reagent. 0.75 N NaOH is carefully stirred in, to a permanent pink with phenol red (about 3 ml are required). When a normal thyroid gland is to be analyzed, it is homogenized in water or dilute  $H_2SO_4$ , the total volume of suspension plus rinsings being 100 ml. 1 ml of the thoroughly mixed suspension is taken as an aliquot and is added to 2 ml of dog plasma stock in a 50 ml centrifuge tube. The Somogyi zinc precipitation is carried out as usual, followed by the four washings. In this case, the PI value of the dog plasma must be determined and deducted, together with the reagent blank value.

The four washings described have been found adequate to eliminate 99.92 per cent of 1000  $\gamma$  of inorganic iodide added per 100 ml of plasma and thus should offer ample routine protection. With the same procedure 5  $\gamma$  per cent of added thyroxine iodine are retained completely by the precipitate, and 5  $\gamma$  per cent of added diiodotyrosine iodine are retained to 75 per cent of completion. This curious situation has previously been noted by Man and coworkers (2).

*2 Digestion*—The amount of chromic acid has been increased over that recommended by Taurog and Chaikoff in order to insure an adequate excess for lipemic plasmas as well as for proteins of other tissues. Potassium or sodium dichromate can be used as well as chromic acid, but they are so much less soluble that the amount to be added would need to be used in solid form. Care should be taken that the second digestion is not prolonged beyond the stage of definite appearance of fumes, since excessive heating often results in considerable loss of the iodine present.

*3 Distillation*—In this laboratory the digestion of organic material has usually been carried out in ordinary 250 or 300 ml Pyrex Florence flasks so that many determinations could be carried out without the expense entailed in an equal number of the special flasks used on the distillation apparatus. The principal inconvenience resulting from this economy is the insertion of an extra transfer from digestion flask to distillation flask by means of the 25 ml of water, used in 5 ml portions.

10 per cent sodium sulfite is used as the absorbing solution in the trap as a simple substitute for the  $Na_2CO_3$ - $NaHSO_3$  combination utilized by Talbot *et al*. Results in this laboratory with NaOH alone, as proposed by

Tanog and Chaikoff, have shown uniformly unsatisfactory recoveries, of the order of 0 to 10 per cent. The need for a reducing solution suggests that the volatile iodide may actually be iodine, instead of HI.

The duration of the distillation period is a compromise between the desire for a 100 per cent recovery and for as short a reaction time as possible. In one series of determinations, the recoveries of 0.10  $\gamma$  of inorganic iodide added to digests of dog plasma were found to be as follows for various times: 5 minutes, 56 per cent, 7½ minutes, 78 per cent, 10 minutes, 85 per cent, 12.5 minutes, 89 per cent, 15 minutes, 92 per cent. At that rate, about 25 to 30 minutes would be required for a 98 to 100 per cent complete distillation. Since other series with 0.05 to 0.25  $\gamma$  of I have shown 87 to 95 per cent recoveries in 10 minutes, this time has been selected. A correction factor covering the 5 to 15 per cent loss may be introduced if desired.

Although the sodium sulfite has been found essential, as noted earlier, it must be decomposed to SO<sub>2</sub> by acid and thus blown off in order not to cause a complete and non-specific decolorization of the ceric solution later. Compressed air has been used, since the substitution of N<sub>2</sub> did not alter the results. A simple and effective set-up consists of a four or six outlet manifold, with finely drawn out glass tubing which reaches nearly to the bottom of each tube. The tubes specified are long enough so that vigorous bubbling can be accommodated. A screw clamp control should be provided for each nozzle to prevent excessive air currents. This aeration step also results in some volatilization of water, so that the level in each tube should be less than 25 ml. even after adequate rinsing of the fine glass tubes. Thus, ample allowance is made for the addition of water to a consistent final volume.

4 *Colorimetric Determination of Iodide*—This part of the determination is the one most apt to present difficulties, owing to the need for rigorous control of nearly every phase. However, with the requisite amount of care, highly dependable results can be obtained, as is shown by Fig. 1.

Time must be controlled to a far greater extent than is usual with colorimetric procedures, inasmuch as differing rates of a reaction eventually proceeding to completion are involved. Those who have previously used the ceric sulfate-arsenious acid reaction have taken two, and often more, readings in order to establish definitely the rate of decolorization. However, in the interests of simplification, it has seemed desirable to acknowledge the arbitrariness of all colorimetric procedures and to make only a single routine reading at 15 minutes. This should be supplemented by a 30 minute reading whenever the first reading shows a decolorization amounting to less than 40 units on the Klett-Summerson colorimeter (yielded by 0.005  $\gamma$  of I) if one desires the most accurate results possible in the lower range of iodide values.

Since accurate temperature control is also necessary during the incubation, it has been found convenient to employ a test-tube rack of appropriate size suspended from hooks hung on the opposite sides of a constant temperature water bath maintained at 37°. Although this was done to avoid the nuisance of continually resetting the thermoregulator, any temperature in the region of 30–40° is satisfactory, provided it is regulated within  $\pm 0.1^\circ$ .

The ceric ammonium sulfate solution is measured with the greatest possible accuracy, since it contributes the initial amount of color. The 0.5 ml required is delivered from a fine tipped, small orifice pipette, between two marks. There is little need for accurate standardization of the solution from an oxidation-reduction standpoint, because each new stock

TABLE I

*Effect of Sodium Chloride on Iodide Catalysis of Ceric Sulfate Decolorization*

Iodine	NaCl mg						
	0 0	1 0		2 0		3 0	
	Colorimeter reading	Colorimeter reading	Increase*	Colorimeter reading	Increase*	Colorimeter reading	Increase*
$\gamma$			per cent		per cent		per cent
0	490	488		486		485	
0 010	445	422	55	410	82	411	82
0 025	359	319	35	304	47	303	47
0 050	241	199	23	184	34	174	35
0 100	86	50	27	43	31	43	31

\* This was calculated on the basis of the iodine values of the colorimeter readings without NaCl

will require that a blank be established and that the iodine effect be standardized

The 45 second interval between tubes has proved the most valuable, 30 seconds being too short an allowance for all colorimeter readings to be taken and 60 seconds unnecessarily long. One control blank in duplicate plus nine determinations in duplicate on distillates (twenty tubes in all) can thus be handled successfully in each series. The photoelectric colorimeter to be used should be adequately tested for adjustment immediately before one starts the addition of ceric sulfate.

Table I shows the considerable enhancement of the iodide catalysis caused by the inclusion of 1 to 3 mg of sodium chloride per tube. The maximum effect appears to be exerted on the smaller amounts of iodide, being as great as 82 per cent with 0.010  $\gamma$  of iodine. Quite satisfactory, reproducible results can thus be obtained with 0.005  $\gamma$  of I. That contamination of the salt with iodide is not giving a spurious "catalysis" is evident

from inspection of the data in Table I the blank values on the reagents show only a slight extra decolorization with increased amounts of chloride, the results with 20 and with 30 mg of added NaCl are essentially the same

### Results

Although the standardization of the iodide-catalyzed ascorbic acid decolorization of the yellow ceric solution requires the extraordinary

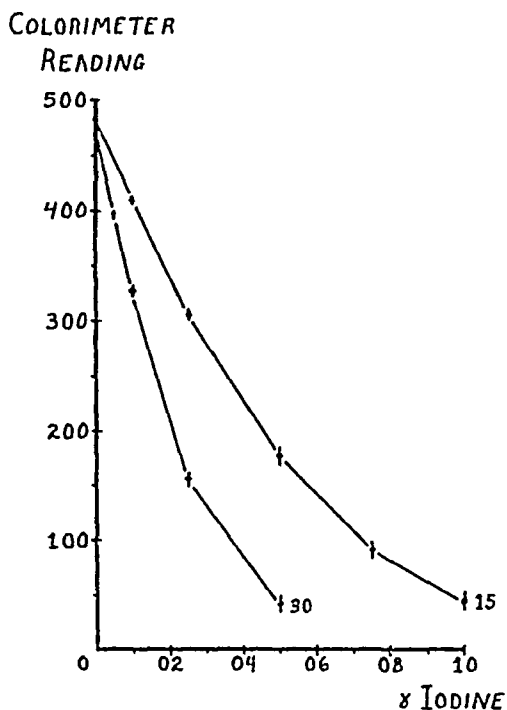


FIG 1 Relationship between the Klett-Summerson colorimeter reading and standard amounts of iodine. The incubation time is indicated on each curve as 15 or 30 minutes

precautions already discussed, remarkably consistent results are obtained when these precautions are scrupulously observed. Fig 1 shows the mean and the maximum deviation from the mean of points on a calibration curve, ten duplicate determinations were performed on each point over a period of 3 months. From the 30 minute curve in Fig 1 it can be seen that reliable results have even been obtained with 0.005  $\gamma$  of iodide under these circumstances.

In order to test the application of this sensitive analytical procedure to biological material, aliquots of dialyzed, pooled dog plasma were analyzed as such and after the addition of amounts of iodide, thyroxine, or diiodo-tyrosine ranging from 0.05 to 0.50  $\gamma$  (as I). Table II shows that recoveries were 87 to 95 per cent complete when the addition was made before dis-

tillation was carried out, whereas they were 98 to 101 per cent when iodide was added to the distillate. These findings strongly indicate some loss of iodide during distillation, probably due to the arbitrary termination of

TABLE II  
*Recovery of Iodine from Compounds Added to Dog Plasma*

Substance added	Added amount	Amount recovered*	
		$\gamma$	per cent
NaI, before digestion	0 10	0 088	88 0
" " "	0 25	0 226	90 4
Thyroxine, before digestion	0 10	0 093	93 0
" " "	0 20	0 181	90 5
Duodotyrosine, before digestion	0 10	0 090	90 0
NaI, after digestion	0 05	0 045	90 0
" " "	0 10	0 092	92 0
" " "	0 25	0 217	86 8
" " "	0 50	0 439	87 8
KIO <sub>3</sub> , " "	0 10	0 085	85 0
" " "	0 25	0 236	94 4
NaI, " distillation	0 10	0 101	101 0
" " "	0 25	0 244	97 6

\* All figures are the averages of at least four duplicate determinations

TABLE III  
*Protein-Bound Iodine in Tissues of Various Species*

Species	Tissue	No. of animals	Protein bound iodine
			$\gamma$ per 100 ml
Rat, normal	Blood plasma	28	4 2 $\pm$ 0 4*
" on thiouracil	" "	23	1 1 $\pm$ 0 2
" " thyroxine	" "	15	18 0 $\pm$ 1 0
Dog, normal	" "	8	2 3
Human, normal	" "	6	7 5
Rat, normal	Thyroid	7	23 3†
" " "	Liver	2	30 6
" " "	Kidney	2	35 5
" " "	Heart	2	26 6
" " "	Skeletal muscle	2	26 7

\* Standard deviation

† Micrograms of PI in the entire thyroid gland (10 to 20 mg)

this step at 10 minutes. As previously mentioned, it was not thought worth while to continue the distillation to the point of a more complete recovery. In this laboratory, a 10 per cent correction is routinely made.

Another fact which should be pointed out is the poor recovery of inorganic iodide in the absence of organic material to be digested. Re-

coveries under such circumstances range from 70 to 85 per cent. Taurog and Chaikoff also noted this, and reported the use of iodine-free wheat as an organic carrier. In this laboratory we have found dialyzed dog serum much more convenient, it can be stored in the frozen condition for long periods of time or it can be lyophilized and redissolved when needed. The iodine values are low and remain stable over long periods of storage in the frozen or dried state.

Studies on the various solutions obtained at different stages of the procedure have revealed unexpected keeping qualities, provided bacterial contamination does not occur. Plasmas and final distillates have been kept in the refrigerator without preservative for as long as 3 months without a detectable loss of iodide. Simple water solutions of NaI containing as little as 0.01  $\gamma$  of I per ml. have remained stable for 10 months.

Table III contains the results of estimations of protein-bound iodine in blood plasma of three species, as well as in various tissues of the rat. In all instances, inorganic iodide was washed out. When thyroxine or diiodotyrosine was added in amounts equivalent to 1.0 to 5.0  $\gamma$  per 100 ml. of plasma before the washing, recoveries of approximately 100 and 75 per cent (corrected) respectively were obtained. These findings indicate adsorption of thyroxine and, to a somewhat lesser extent, diiodotyrosine on the zinc proteinate precipitate. The binding must be strong to withstand four washings.

#### SUMMARY

A procedure has been reported for the determination of protein-bound iodine in various tissues, including blood plasma. The principal steps are precipitation, washing and then oxidation of the protein, distillation of the iodine, and colorimetric determination of the iodine by means of its catalytic effect on the reduction of ceric ions by arsenious acid.

The present method, combining and modifying previous methods, permits a satisfactory analysis to be performed on 2 ml. of plasma, one-hundredth of a normal rat thyroid gland, or 500 mg. of rat liver, kidney, heart, or skeletal muscle.

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# THE SYNTHESIS OF FATTY ACIDS IN ADIPOSE TISSUE IN VITRO

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The conversion of carbohydrate into fatty acids in the animal body has been clearly demonstrated by Schoenheimer and Rittenberg (1) and by Bernhard and Schoenheimer (2) with the aid of deuterium oxide. The  $D_2O$  concentration of the body fluids was raised and the deuterium introduced into the fatty acids was taken as a measure of the newly synthesized fatty acids.

It is generally assumed that the liver is the sole site of this synthesis. However, a quantitative analysis made by Stetten and Grail (3) is not easily reconciled with this assumption. According to their results, it has been calculated that the quantity of fatty acids synthesized per day by a mouse is about 4 times the quantity present in the liver. If the liver is assumed to be the major site of synthesis, the half life time of fatty acids in the liver should be several hours, whereas the half life time actually found was between 26 and 28 days. Tepperman, Brobeck, and Long (4) produced evidence that carbohydrate can be converted into fat in the extrahepatic tissues. They were able to show that fatty acid synthesis was augmented in rats on a dietary training, and that this augmentation persisted in the functional absence of the liver.

Tuerkischer and Wertheimer (5) suggested that adipose tissue may play a part in the synthesis of fat. They showed that if, after rats have been fasted or given inadequate diets long enough to exhaust their stores of fat, the animals are realimented with high carbohydrate diets, glycogen is initially deposited in the fat cells. Only after an interval has elapsed does the glycogen gradually disappear, giving way to fat. In earlier experiments (6), it had been found that in all conditions favoring fat synthesis from carbohydrates, fat deposition in adipose tissue is preceded by the appearance of glycogen in this tissue. It was furthermore shown by Mirski (7) that isolated, glycogen-containing adipose tissue has a respiratory quotient of 1.1 to 1.3, whereas ordinary, glycogen-free adipose tissue has a respiratory quotient of 0.70. The respiratory quotient of glycogen-laden adipose tissue was especially high and most consistent when the tissue was incubated in serum. It is thus presumable that adipose tissue is capable of synthesizing fatty acids.



In order to test this assumption, the method of Schoenheimer and Rittenberg (1) was used with adipose tissue incubated *in vitro*. Deuterium oxide was added to the medium and the deuterium found in the isolated fatty acids was taken as a measure of synthesis of fatty acids. Experiments were carried out with mesenteric fat, interscapular fat, and gon fat of rats on dietary régimes inducing fat synthesis and deposition of glycogen in adipose tissue, on the one hand, and of rats on a stock diet, on the other hand.

TABLE I  
*Introduction of Deuterium into Fatty Acids of Adipose Tissue*

Tissue	Treatment of rats	Fatty acids per 100 gm body weight	Deuterium concentration	
			Atom per cent excess in fatty acids	Per cent ratio in fat to that in medium
		mg		
Mesenteric fat	Stock diet	145	0.020	0.22
" "	" "	180	0	0
" "	Diet 1	130	0.022	0.23
" "	" 1	122	0.025	0.27
" "	" 2	95	0.046	0.51
" "	" 2	105	0.040	0.44
Interscapular fat	Stock diet	85	0.034	0.38
" "	" "	80	0.052	0.57
" "	Diet 1	70	0.064	0.71
" "	" 1	75	0.056	0.62
" "	" 2	70	0.021	0.23
" "	" 2	68	0.074	0.81
Gon fat	Stock diet		0.012	0.13
" "	Diet 1		0.018	0.20
" "	" 2		0.023	0.25
" "	" 2		0.019	0.21
Liver slices*	Diet 1	88	0.062	1.75
" "	" 2	82	0.070	2.00

\* Liver slices were incubated in serum with only 5 per cent deuterium oxide, making a final deuterium concentration in the tissue fluid of about 3.5 per cent.

#### EXPERIMENTAL

Adipose tissue was taken from rats on a stock diet and from rats maintained on the following dietary régimes. In Diet 1 food intake was restricted for 5 to 7 days until a weight loss of 20 per cent resulted, followed by subsequent feeding *ad libitum* for 48 hours on a diet consisting of 70 per cent carbohydrates, 20 per cent casein, and 10 per cent oil. Diet 2 differed from Diet 1 only in that rats were kept on the restricted diet until 30 per cent weight loss resulted and were then refed for 16 hours only. Adipose tissue

of the rats on Diets 1 and 2 contained glycogen in a concentration of 0.2 to 0.5 per cent

Tissues were removed, finely cut with scissors, and incubated for 4 hours at 37° in 3 volumes of rat serum enriched with deuterium oxide to a concentration of about 10 atom per cent. After incubation, the fat was saponified with 20 per cent KOH, acidified, and the fatty acids extracted with ether. After evaporation of the ether, the residue was dissolved in aqueous 5 per cent KOH (in order to eliminate the carboxyl deuterium), acidified, and re-extracted with ether. The ether was dried with sodium sulfate and evaporated. The deuterium concentration in the fatty acids thus isolated was estimated by the method of Keston, Rittenberg, and Schoenheimer (8).

### Results

The results, summarized in Table I, show that deuterium is introduced into the fatty acids of adipose tissue incubated in serum enriched with deuterium oxide. The deuterium concentration is generally higher with adipose tissue from rats in a condition of accelerated fat synthesis (Diets 1 and 2) than with adipose tissue from normal rats. With normal groin fat, the deuterium concentration found in the fatty acids was very low, owing to the high initial fat content. Higher values are found when part of the fat has previously been depleted (Diet 2).

The experiments were carried out in serum as a medium, since in this medium the most consistently high respiratory quotients were found (7). Several experiments carried out in Ringer's solution gave similar but less marked results.

### DISCUSSION

The results confirm the assumption that adipose tissue is capable of synthesizing fatty acids. The higher activity found in glycogen-containing adipose tissue (Diets 1 and 2) suggests that glycogen may play a rôle in the synthesis of fatty acids. The results do not as yet permit an exact evaluation of the part played by adipose tissue in fat synthesis in the animal body. When adipose tissue and liver are compared, it is found that liver is more active in renewing its fatty acids by synthesis. Since, however, the total amount of fatty acids in adipose tissue is many times greater than that in liver, adipose tissue may be responsible for a high percentage of the total quantity of fat synthesized in the body, in spite of the slower overturn in this tissue.

### SUMMARY

Adipose tissue incubated *in vitro* in serum enriched with deuterium oxide introduces deuterium into its fatty acids. The rate of introduction is greater in adipose tissue of rats on a diet accelerating fat synthesis in the body.

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# GLUTAMINE, AN ANTIMETABOLITE FOR STAPHYLOCOCCUS AUREUS

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In a previous communication (1) it was shown that the antibacterial effect of L- and DL-N-( $\gamma$ -glutamyl)-ethanolamine and DL-N-( $\gamma$ -glutamyl)-ethylamine upon *Staphylococcus aureus* could be reversed by glutamic acid. Preliminary data (1) showed that glutamine was unable to prevent this inhibition. The ineffectiveness of glutamine in overcoming the inhibition seemed to be rather curious, since glutamine is known to be a growth factor for at least two organisms, *Streptococcus pyogenes* (2, 3) and *Neisseria gonorrhoeae* (4), and since it has furthermore recently been shown to counteract the inhibition of growth of *Lactobacillus arabinosus* caused by a sulfoxide derived from methionine (5, 6).

Further experiments were therefore undertaken to reinvestigate this problem. Data will be presented to show that glutamine, alone, very markedly inhibits the growth of our previous test organism, *Staphylococcus aureus*, and that this inhibition is specifically reversed by L-glutamic acid.

## EXPERIMENTAL

**Methods**—We followed the technique previously described (1) with respect to the composition of the medium, the test organism, incubation periods, and growth estimation. We used as an inoculum 0.1 ml per tube of a 100-fold dilution of a barely turbid suspension of staphylococci. Of the test materials used, glutamine and glutathione were sterilized by filtration and others by autoclaving. The sterile ingredients were added aseptically to the medium, and the volume was made up to 5 ml with sterile water. Growth was measured turbidimetrically with an Evelyn type electrophotometer. Since the experiments were carried out in specially matched tubes, the estimation of turbidity could be made at any stage of growth, and our final measurement was made after 48 hours, more prolonged incubation did not alter the results.

**Effect of Glutamine on Staphylococcal Growth**—Table I shows that glutamine inhibits the growth of *Staphylococcus aureus* to a very marked degree. As little as 3 mg of glutamine per 5 ml of medium inhibits growth by 80 to 90 per cent, and an inhibition of about 50 per cent is caused by 2 mg. Lower concentrations produced less marked inhibition, whereas

higher ones did not produce complete bacteriostasis. A similar effect was found with the glutamic acid alkylamines (1). However, glutamine is 4 to 5 times more potent than the two alkylamines, L- and DL-N-( $\gamma$ -glutamyl)-ethanolamine and DL-N-( $\gamma$ -glutamyl)-ethylamine, previously tested.

Since the glutamine preparation used is not a synthetic product and may therefore be contaminated with biologically active substances such as arginine (7), it seemed necessary to determine whether the inhibitory effect was really due to glutamine itself or to a possible associated by-product. Experiments were carried out to rule out the latter possibility. (1) Different batches of glutamine, namely two batches of glutamine from General Biochemicals, Inc., as well as a batch of glutamine from The British Drug

TABLE I  
*Effect of Glutamine on Growth of Staphylococcus aureus*

Compound added	Concentration	Photometer reading after 48 hrs. growth*
	mg per 5 ml	
None		45
"		46
Glutamine	0.5	46
"	1.0	52
"	2.0	64
"	3.0	78
"	4.0	88
"	5.0	90
"	10.0	87
None (uninoculated)		100

\* A reading of 100 indicates no growth. Increased growth is reflected by a decreased reading.

Houses, Ltd., gave identical results. (2) Arginine had no effect whatsoever. (3) The inhibition is of a competitive character and is specifically counteracted by L-glutamic acid. An almost constant ratio was found to exist between the inhibitor (glutamine) and the metabolite (glutamic acid) (Table II).

We are thus obviously dealing with a specific inhibition due to glutamine.

*Specificity of L-Glutamic Acid in Reversing Glutamine Inhibition*—We next attempted to elucidate the mechanism by which glutamine inhibits the growth of *Staphylococcus aureus*. A series of compounds structurally related to L-glutamic acid was chosen. L-asparagine and the sodium salts of DL-aspartic acid, DL- $\alpha$ -aminoadipic acid, and L-pyrrolidonecarboxylic acid. The results are summarized in Table III. It may be seen that, although some of these substances stimulated the growth of the organism

to a slight degree, none of them, except L-glutamic acid, was capable of abolishing the inhibition caused by glutamine

The specificity of L-glutamic acid as an antagonist of glutamine is even more pronounced than would appear from the above. When the natural optical isomer is replaced by the racemic DL-glutamate, the latter, instead of showing the expected 50 per cent activity, as compared with the L antipode, was found to be completely inactive (Table III). This experiment was repeated several times with various preparations of DL-glutamic acid (Merck and Company, Inc., Hema Drug Company, Inc., and a sample

TABLE II

*Inhibition of Growth of Staphylococcus aureus by Glutamine, and Its Reversal by L-Glutamic acid*

Glutamine concentration	L-Glutamic acid concentration	Photometer reading after 48 hrs growth	Glutamine L Glutamic acid at 50 per cent inhibition
mg per 5 ml	mg per 5 ml		
0	0	47	
0	4	45	
4	0	86	
4	0.2	86	
4	0.5	72	8
4	1.0	50	
8	0	86	
8	0.5	85	
8	1	77	6 ca
8	2	67	
8	3	48	
12	0	87	
12	1	82	
12	2	71	6 ca
12	3	68	
12	4	56	

prepared by Dr N Lichtenstein, The Hebrew University, Jerusalem). The same results were regularly obtained even when concentrations 5 times as great as the effective concentration of L-glutamic acid were used. DL-Glutamic acid does not actually inhibit growth, but is merely ineffective in restoring growth in the presence of the inhibitor (glutamine).

*Effect of Pteroylglutamic Acid and Glutathione on Inhibition of Growth by Glutamine*—Since none of the compounds related to L-glutamic acid was able to reverse the inhibition caused by glutamine, we tried the effect of two natural tripeptides containing L-glutamic acid as a component, (1) pteroylglutamic acid (folic acid), in which the glutamic acid is bound through its amino group, and (2) glutathione, in which the glutamic acid is

bound through its  $\gamma$ -carboxyl, as in glutamine and in the  $\gamma$ -glutamyl-  
amines Table IV shows that both tripeptides are highly efficient in re-

TABLE III

*Effect of Various Compounds Related to Glutamic Acid on Inhibition of Growth of  
Staphylococcus aureus by L-Glutamine*

Inhibitor	Glutamic acid* and related compounds	Concen- tration	Photometer reading after 48 hrs growth
		mg per 5 ml	
None	None		43
"	L-Glutamic acid	5	43
"	DL-Glutamic "	5	43
"	" "	15	46
"	DL-Aspartic "	5	42
"	" "	15	47
"	L-Asparagine	5	37
"	"	15	41
"	DL- $\alpha$ -Aminoadipic acid†	5	42
"	" "	15	41
Glutamine, 5 mg per 5 ml	None		87
" "	L-Glutamic acid	0.5	65
" "	" "	2	42
" "	" "	5	38
" "	DL-Glutamic "	5	85
" "	" "	10	82
" "	" "	15	78
" "	DL-Aspartic "	5	82
" "	" "	15	85
" "	L-Asparagine	5	82
" "	"	15	85
" "	DL- $\alpha$ -Aminoadipic acid	5	81
" "	" "	15	83
" "	L-Pyrrolidonecarboxylic acid	10	84
" "	" "	10)	79
	Ammonium chloride	10)	

\* All the acids were assayed as neutral sodium salts

† We are indebted to Dr Heinrich Waelsch, Columbia University, New York,  
for the sample of DL- $\alpha$ -aminoadipic acid

versing the inhibitory effect of glutamine On the basis of glutamic acid  
content, folic acid (33 per cent glutamic acid) is about 15 to 20 times as  
active as L-glutamic acid and glutathione (48 per cent glutamic acid) is  
about 10 to 12 times as effective as L-glutamic acid

## DISCUSSION

Glutamic acid plays an important rôle in the metabolism of *Staphylococcus aureus*. This organism grows luxuriantly in a synthetic amino acid medium devoid of glutamic acid, apparently synthesizing the required amino acid (8). If glutamic acid is added to the medium, the staphylo-

TABLE IV

*Effect of L-Glutamic Acid, Pteroylglutamic Acid, and Glutathione on Inhibition of Growth of Staphylococcus aureus by Glutamine*

Inhibitor	Glutamic acid and derivatives	Concentration  mg per 5 ml	Photometer reading	
			24 hrs	48 hrs
None	None		51	40
"	"		54	42
"	L-Glutamic acid	2	66	42
"	Pteroylglutamic acid*	0.5	63	39
"	Glutathione	1	61	43
Glutamine, 4 mg per 5 ml	None		85	84
" "	L-Glutamic acid	0.1	82	83
" "	" "	0.3	84	84
" "	" "	0.6	75	63
" "	" "	1	62	48
" "	" "	2	55	39
" "	Pteroylglutamic acid	0.03	80	78
" "	" "	0.1	75	57
" "	" "	0.3	73	42
" "	" "	0.5	72	41
" "	Glutathione	0.03	87	84
" "	"	0.1	82	60
" "	"	0.3	76	51
" "	"	0.6	76	47
" "	"	1.0	71	42

\* Synthetic pteroylglutamic acid (folic acid) has been obtained through the courtesy of Dr S. M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

cocci assimilate it and concentrate it in their protoplasm (9). Our strain behaved normally in this respect in that it was capable of building this amino acid by itself. Now, if sufficient glutamine is added to a casein hydrolysate medium which contains glutamic acid, the growth is inhibited, and extra glutamic acid is required to overcome the inhibitory effect. This inhibition is very specific. Neither the 4-carbon analogues, DL-aspartic acid and L-asparagine, nor the 6-carbon analogue, DL- $\alpha$ -amino-



dipic acid, has any effect upon it. L-Pyrrolidonecarboxylic acid and its ammonium salt are also without effect. Furthermore, the racemic DL-glutamic acid, though not in itself inhibitory, is incapable of counteracting the inhibition due to glutamine.

The ineffectiveness of DL-glutamic acid is striking. Instead of showing the expected 50 per cent activity, it is not effective at all. The inactivity of racemic glutamic acid may be explained in either of two ways. The D antipode may show inhibiting properties comparable in extent to the counteracting effect of the L antipode on glutamine, and these two effects may cancel each other. It is possible, also, to assume the formation of a stable racemate, the antipodes of which have greater affinity for each other than that of the enzyme for the L form (10). This problem is now under investigation.

L-Glutamic acid and the two natural tripeptides, glutathione and folic acid, are the only ones in a series of substances tested which reverse the inhibition of staphylococcal growth by glutamine. In folic acid, the glutamic acid is bound through its amino group, whereas in glutathione it is bound through the  $\gamma$ -carboxyl as in glutamine and in the  $\gamma$ -glutamylamines. It is, therefore, rather surprising that glutathione is so efficient in overcoming the inhibition by glutamine. It is interesting to note that Waelsch, Owades, Miller, and Borek (6) also observed that glutathione behaves like glutamic acid and not at all like glutamine towards the sulphoxide of methionine.

These results may best be interpreted by assuming that we are dealing with an inhibition of the competitive type. Glutamine, closely related to glutamic acid, possesses great affinity for that enzyme system which normally combines with glutamic acid. Since the staphylococci are apparently devoid of glutaminase, the glutamine is not broken down, and reactions essential for glutamic acid metabolism and growth are thereby blocked. The resulting inhibition is, however, completely reversible, since growth is resumed on the addition of glutamic acid even if bacteriostasis has already taken place.

The fact that glutamine is an efficient antimetabolite seems remarkable to us. The great majority of the numerous structural analogues which show antagonistic properties towards their mother substances are artificially changed compounds. Some substances, however, are naturally occurring metabolites (*e.g.* asparagine, which is antagonistic to  $\beta$ -alanine in a yeast (11), isoleucine antagonistic to leucine in *Pasteurella pestis* (12), and arginine antagonistic to lysine in *Neurospora* (13)).

Furthermore, our data illustrate the fact that under certain conditions an essential metabolite and growth factor may show inhibitory properties. The inhibiting concentration of glutamine is, however, higher than that

required for growth stimulation in sensitive organisms, ranging as it does from 0.06 mg per 100 ml in *Lactobacillus casei* (14) to 0.03 mg and even to 20 mg per cent in *Streptococcus hemolyticus* (2, 3), whereas an inhibition of about 50 per cent is obtained only by 40 mg per cent. Nevertheless, in spite of the high concentrations of the glutamine this is a specific inhibition governed by the laws of competitive interference with the essential metabolite.

The question of the identity of the metabolite (whether it is the glutamic acid itself or some glutamic acid peptide) also merits mention. Since both glutathione and folic acid are considerably more effective than glutamic acid in overcoming the growth inhibition produced by glutamine, one might infer that these two substances are actually the end-products of glutamic acid metabolism, the formation of which is inhibited by glutamine. This inference is to a certain extent, though not conclusively, contradicted by the data in Table IV. Each of these three substances, L-glutamic acid, folic acid, and glutathione, when added to the assay medium, causes a prolonged lag phase, a transient inhibition which disappears on further incubation (compare 24 and 48 hour data in Table IV). The possibility is therefore not excluded that glutamic acid is converted not into one of these peptides but into another substance of similar structure.

#### SUMMARY

The inhibiting effect of glutamine upon *Staphylococcus aureus* is demonstrated. This inhibition is of a competitive type and is specifically reversed by L-glutamic acid, pteroylglutamic acid, and glutathione. Substances like DL-aspartic acid, L-asparagine, DL- $\alpha$ -aminoadipic acid, and L-pyrrolidonecarboxylic acid are ineffective.

The unexpected ineffectiveness of DL-glutamic acid in overcoming the inhibition is discussed.

The inhibition indicates the absence of a glutaminase capable of converting glutamine into glutamic acid.

The role of pteroylglutamic acid and glutathione in the metabolism of *Staphylococcus aureus* is briefly discussed.

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# THE DISTRIBUTION AND EXCRETION OF INJECTED URANIUM\*

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The distribution and excretion of injected uranium have been the subject of a number of papers (1-9) wherein a variety of analytical methods was employed and a variety of conclusions reached. For example, some authors (6, 8) considered the liver as an important site of uranium deposition, while others (3, 4) reported the liver to be uranium-free. Some (1, 3) found uranium to be excreted in the bile, a fact denied by others (7).

All of these investigators agreed that uranium accumulates in the kidneys and most of the recent publications indicated that large quantities of injected uranium were excreted in the urine. None of these investigators<sup>1</sup> examined bone as a possible site of storage, in spite of the fact that Hoffmann (10) found traces of uranium in the bones of normal, untreated animals.

In the experiments reported here, balance studies were performed on rats, in addition, corollary studies with other species were conducted to clarify certain points. Injected uranium was found to be rapidly excreted in the urine, very little appearing in the feces. Bone and kidney were the only tissues which retained significant quantities.

## EXPERIMENTAL

### *Methods*

Because of the influence diet might have on the distribution and excretion of uranium, a synthetic ration, which could be defined and reproduced, was

\* This paper is based on work performed under contract No W-7401-Eng-49 for the Manhattan Project at the University of Rochester.

<sup>1</sup> Exceptions can be found in the classified literature of the Manhattan Project. A comprehensive study of the distribution and excretion of injected and ingested uranium was reported by Tannebaum, A., Silverstone, H., and Koziol, J., Chicago Atomic Energy Report No CH-3650, similar experiments with uranium given by intratracheal insufflation were reported by Thompson, H. E., and Steadman, L. T., Rochester Atomic Energy Report No M-1984. The results of these independent studies are in excellent agreement with the data reported here.

chosen. Rats kept on this ration for several months grew well and evidenced no deficiency symptoms.

This diet had the following composition, in parts by weight: 25 casein, 40 starch, 10 sugar, 5 yeast, 4 alfalfa, 20 Cellu flour, 7.5 lard, 2.5 cod liver oil, 4.9 salt mixture (11), 1.0 calcium carbonate, 0.1 Natola (fish oil concentration), 0.2 choline chloride, and 0.21 vitamin mixture.

The vitamin mixture had the following composition in mg: thiamine 80, riboflavin 160, pyridoxine 120, nicotinic acid 250, calcium pantothenate 250, *p*-aminobenzoic acid 230, inositol 500, and copper sulfate 500.

The high Cellu flour content of the diet initially produced a mild diarrhea lasting 1 or 2 days. Because of this, all experimental rats were given a minimal equilibrating period of 1 week on the ration before uranium was administered.

Male and female Wistar rats weighing  $200 \pm 5$  gm. were injected intravenously (by tail vein) with a solution of 0.1 per cent uranyl nitrate (hexahydrate) at a dose level of 2.5 mg. of U per kilo. The animals were placed in individual, all-glass metabolism cages which permitted separate urine and feces collection. Each day, the cages were washed thoroughly with 2 N HCl solution containing 0.1 per cent detergent (special sample of acid-stable detergent, Igepal CTA extra, kindly furnished by the General Dye-stuff Corporation, New York). After specified intervals of from 45 minutes to 44 days after injection, the animals were anesthetized lightly with ether, blood being collected by aspiration from an axillary pouch made according to Kuhn (12), and were then sacrificed. Dissections were made with scrupulously clean instruments which were kept under a 2 per cent solution of sodium bicarbonate when not in use. The following samples were taken: kidney, urinary bladder, spleen, gonads, heart, lungs, liver, stomach and contents, intestines, leg muscle, skin and hair, femurs and humeri, tibiae, radii, fibulae, ulnae, pelvic girdle, three or four vertebrae, ribs, skull, and tail. The rest of the animal was divided into two samples, soft carcass and bone carcass, each bone was scraped free of adhering flesh. A complete balance record, i.e. uranium administered *versus* uranium recovered, was kept for each rat.

Corollary experiments were conducted on other species. In these studies, the animals were anesthetized and infused with fluids as specified, the bladders were cannulated, and, after a single intravenous injection of uranyl acetate, urine samples were collected over varying periods from 4 to 8 hours.

All analyses for uranium were made by the fluorophotometric method described previously (13).

### Results

*Uranium Content of Normal Tissue*—Two normal, untreated rats were sacrificed, dissected, and analyzed as described above to establish the range

of concentrations of uranium in normal tissue The results are given in Table I

The concentrations observed in normal rats were less than 0.1  $\gamma$  per gm of tissue, in agreement with the results of Hoffmann (10) Actually, it is doubtful whether these data represent the true tissue content, rather, they represent a measure of the extent to which contamination was controlled

In the data to follow, only concentrations greater than 0.1  $\gamma$  per gm of tissue were considered significant

TABLE I  
*Uranium Content of Normal Rat Tissues*

Tissue	Rat 1			Rat 2		
	Tissue wt.	Total U*	U concentration	Tissue wt.	Total U*	U concentration
	gm	$\gamma$	$\gamma$ per gm	gm	$\gamma$	$\gamma$ per gm
Carcass	47.55			71.40	0.00	0.00
Muscle	42.00	0.38	0.01	27.21	0.00	0.00
Skin and hair	33.09	0.45	0.01	31.15	0.39	0.01
Intestines	17.66	0.12	0.01	20.32	0.13	0.01
Liver	8.72	0.22	0.04	9.65	0.00	0.00
Blood	7.03	0.14	0.02	7.77	0.08	0.01
Stomach	4.82	0.04	0.01	1.55	0.02	0.01
Skull	2.71	0.00	0.00	2.97	0.00	0.00
Genitals	2.38	0.00	0.00	2.54		
Lungs	1.15	0.03	0.03	1.03	0.00	0.00
Vertebrae	1.06	0.03	0.03	1.21	0.00	0.00
Tibiae	1.26	0.00	0.00	0.85	0.00	0.00
Kidneys	1.63	0.03	0.02	1.66	0.00	0.00
Femurs	0.79	0.00	0.00	1.01	0.00	0.00
Spleen	1.16	0.03	0.03	1.35	0.01	0.01
Heart	0.81	0.06	0.07	0.82	0.06	0.07
Pelvic girdle	0.67	0.00	0.00	0.75	0.00	0.00
Ribs	0.41	0.03	0.07	0.35	0.00	0.00

\* Total uranium values less than 0.05  $\gamma$  are of doubtful significance (13)

*Analytical Recoveries*—To prevent analytical inaccuracies from giving misleading results in the experiments below, a balance study was conducted on each rat examined. A summary of these results is presented in Table II. Included in the table are the results of control isolations from pure uranyl nitrate solutions carried out simultaneously with isolations from tissue samples.

It should be pointed out that, in these experiments, most errors were positive. For example, of a series of analyses of normal urine specimens which are uranium-free, some will give slightly positive results, some true

blanks, and some slightly negative results. However, all negative results are reported "zero," giving an average which is positive. Therefore, the highest recoveries were observed in those animals from which the greatest number of samples were taken (greatest accumulation of positive errors). In practice, it was the long term animals from which the greatest number of specimens were taken (daily urine and feces collections over periods of 10 to

TABLE II  
*Over-All Recoveries in Balance Experiments*

	No. of samples	Mean recovery	Standard deviation
		<i>per cent</i>	<i>per cent</i>
Uranyl nitrate solutions	38	85	8
Short term animals (14)	400	90	7
Long " " (7)	420	108	13

TABLE III  
*Variation in Uranium Concentration in Rat Blood with Time*

Time after injection	Blood uranium level	
	Females	Males
<i>hrs</i>	<i>γ per ml</i>	<i>γ per ml</i>
0 (Calculated)*	36	36
0 75	1 5	1 3
2 5	0 2	0 0
8		0 1
12	0 1	0 1
24	0 0	0 1
48	0 1	0 0
960	0 0	0 1

\* Calculated on the assumption that the blood volume constituted 7 per cent of the total body weight

40 days). Accordingly, the results obtained from the long term animals have been listed separately in Table II.

The data show that the recovery of uranium from the animal was, in final analysis, equivalent to that obtained with pure solutions of uranyl nitrate.

*Blood*—Analyses of blood have been made on two species, rats and rabbits, the literature contains one report on dogs (8). All three species evidenced a rapid disappearance of uranium from the blood after injection.

The data obtained with rats are summarized in Table III. Fig. 1 presents graphically the results of the experiments on rabbits, including one in which the rabbit had been nephrectomized.

In rats and normal rabbits, over 99 per cent of the administered uranium had left the blood stream in 2 hours. Even nephrectomy did not markedly delay the process. In dogs, as reported by Holman and Douglas (8), uranium left the circulation somewhat more slowly. However, the dogs received uranium intraperitoneally.

*Urinary Excretion*—Data obtained from three species, rats, cats, and rabbits, indicated that the urinary excretion of uranium is also rapid. The averaged urinary excretion of rats is presented in Fig. 2. Most of the ex-

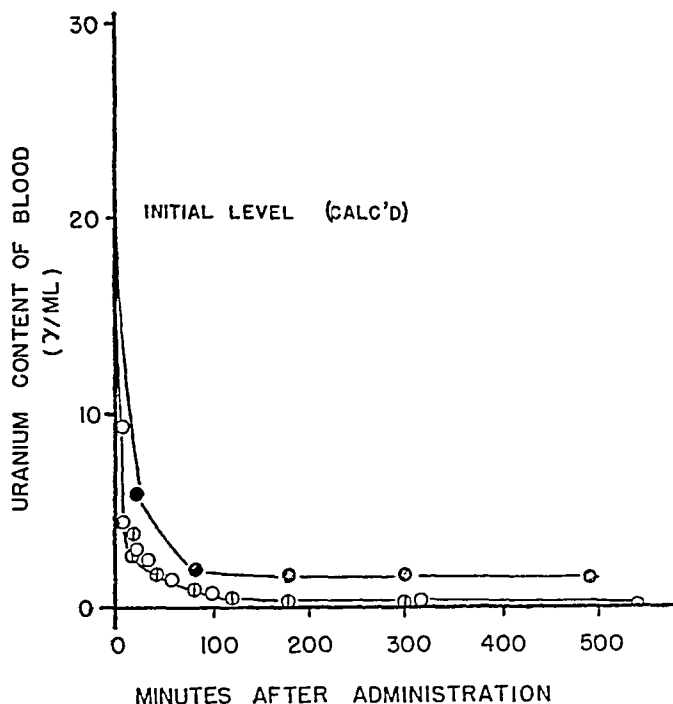


FIG. 1 Disappearance of injected uranium from the blood. O, specimens from normal rabbits, ●, data from a nephrectomized rabbit.

cretion took place in the first 24 hours after administration. Practically all of the excreted uranium was found in the urine. Holman and Douglas (8) obtained similar results with dogs.

Table IV contains the summarized results of studies on cats and rabbits. In these animals, nearly one-fifth of the dose was excreted in 4 hours.

It is interesting that a marked difference was observed between the early urinary excretion of the male and the female rats. This was probably a reflection of differences in the amounts of uranium deposited in the skeleton of the two sexes (see below).



*Effect of Acid-Base Administration*—Work in this laboratory and elsewhere (15, 16) indicated that alkali administration decreased uranium toxicity, as evidenced by mortality. It was not surprising, then, that the simultaneous administration of uranium and sodium bicarbonate resulted

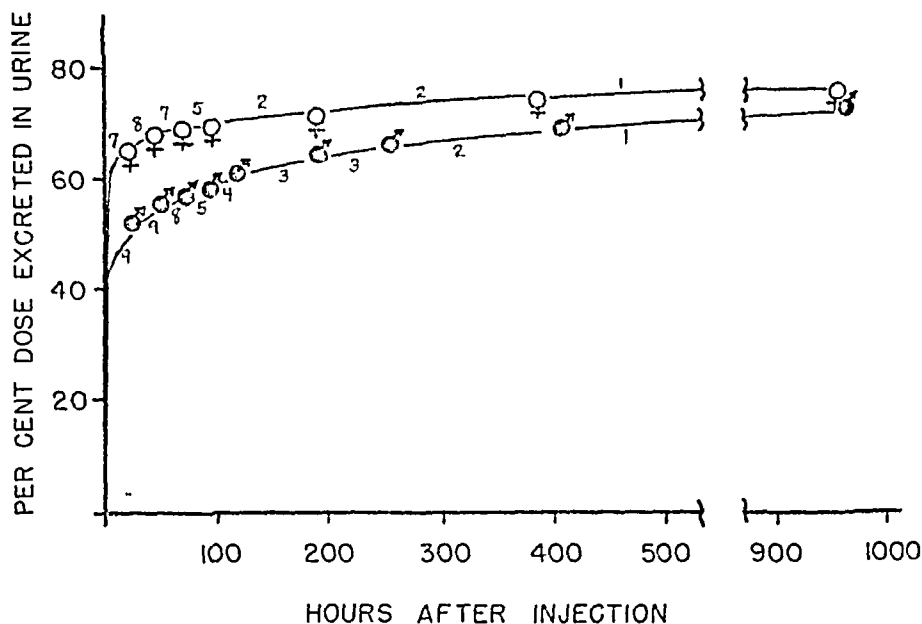


FIG 2 Averaged urinary excretion by male and female rats. The numbers associated with the curves indicate the number of animals averaged.

TABLE IV  
4 Hour Urinary Excretion of Uranium by Individual Cats and Rabbits following Intravenous Injection

Cats		Rabbits	
Dose	Dose in urine	Dose	Dose in urine
mg U per kg	per cent	mg U per kg	per cent
3.4	17.5	1.7	17.8
3.4	19.0	1.7	8.9
6.2	27.4	1.7	14.2
12.1	18.5	5.6	23.2
Average	20.6		16.0

in an increase in the amount of uranium excreted. To illustrate the effects of alkali, representative data have been assembled in Table V.

It is apparent that acidifying substances exerted an effect opposed to that of alkaline substances. Rabbits and cats which were given acid-producing diets excreted a much smaller proportion of the dose.

*Fecal Excretion*—Although the amounts of uranium found in the feces of treated animals were significant, they appear unimportant when compared with the amounts found in the urine. The average total found in the feces

TABLE V

*Effect of Acid Base Administration on  $\frac{1}{2}$  Hour Urinary Excretion of Uranium*

Species	No. of animals	Diet	Infusion fluid 0.85%	Systemic acid base balance	Dose <i>mg U per kg</i>	Dose in urine <i>average per cent</i>
Cat	1	Normal	NaHCO	Alkaline	3.4	65
	5	"	NaCl	Normal	3.4-12.1	24
	2	Meat + NH <sub>4</sub> Cl	"	Acid	3.4	10
Rabbit	1	Normal	NaHCO	Alkaline	5.6	71
	4	"	NaCl	Normal	1.7-5.6	16
	3	Oat	"	Acid	1.7-5.6	3.6

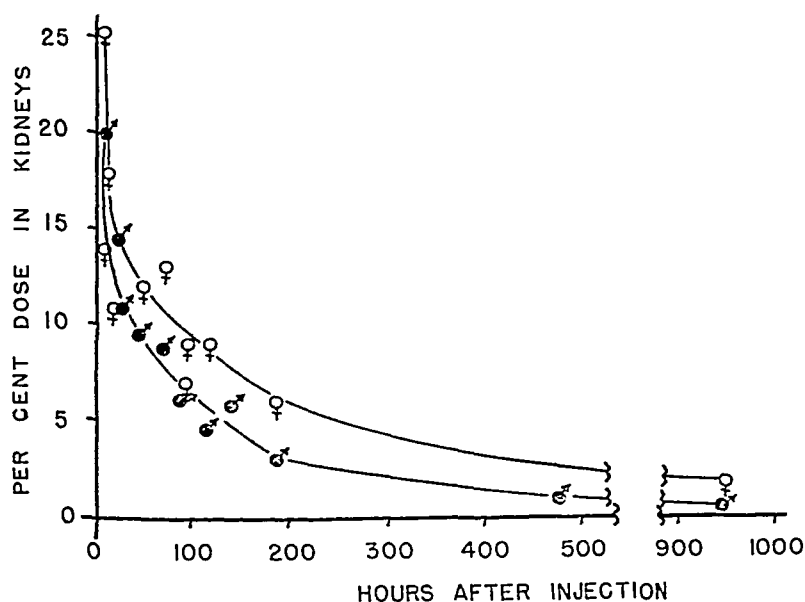


FIG. 3 The relationship between the time after administration and the uranium content of the rat kidney

of twenty-three rats was only 3.9 per cent of the dose. This average includes fecal collections for periods ranging from 0.75 to 960 hours after administration. There was no correlation between the amount of fecal uranium and the time after injection.

The intestines and contents, removed from the carcass, never contained more than 0.5 per cent of the dose. After 2 days, the quantities found in the intestines were insignificant.

There is good reason to believe that the small amounts in the feces did not represent true excretion, but rather contamination. Two possible sources of contamination were (a) direct contamination, the feces rolled down the

TABLE VI  
*Effect of Acid-Base Relations on Uranium Deposition in Cat Kidney*

Diet	Infusion fluid, 0.85%	Dose in kidneys	Dose in urine	Dose in kidney-urine system
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Normal	NaHCO <sub>3</sub>	0.8 at 4 hrs	65	66
"	NaCl	33 " 8 "	20	53
Acid (NH <sub>4</sub> Cl)	"	18 " 4 "	11	62
" "	"	72 " 8 "	7.9	79

TABLE VII  
*Uranium Content of Livers of Rats Given Uranyl Nitrate*

Time after administration	Uranium concentration in liver	
	Males	Females
<i>hrs</i>	<i>γ per gm</i>	<i>γ per gm</i>
0.75	0.5	0.6
2.5	0.5	0.3
8	0.3	
12	0.3	0.6
24	0.3	
48	0.4	0.5
72	0.6	0.6
96	0.3	0.5
120	0.3	0.4
288	0.6	0.1
480	0.1	
960	0.1	0.1

sides of the cage funnel which was wet with previously excreted, uranium-rich urine, and (b) indirect contamination, a few drops of urine (on the 1st day) clinging to the fur would provide the means by which the rats could obtain several per cent of the dose orally.

*Deposition in Kidney*—The deposition of uranium in the kidney was very rapid. The highest kidney values, amounting to one-third of the dose, were noted between 0.75 and 2.5 hours after injection. After 40 days, less

than 2 per cent of the dose remained. As in the case of urinary excretion, sex difference was observed. The data obtained from rats showing rapid mobilization are presented in Fig. 3.

Data illustrating the effect of acid-base balance on uranium deposition in kidney, presented in Table VI, show an inverse relationship between deposition and systemic alkalinity.

*Deposition in Liver*—There was practically no deposition in the liver

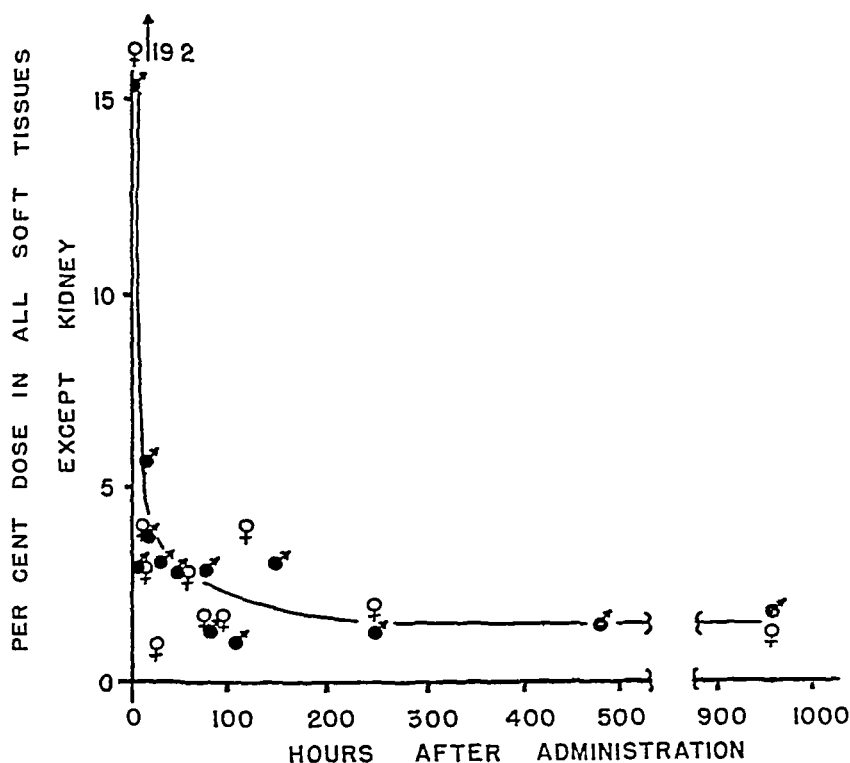


Fig. 4 Disappearance of uranium from the soft tissues

(from 0.1 to 0.9 per cent of the dose), as was indicated by the data from the intravenously injected rats presented in Table VII.

Other species gave similar results. For example, the liver of a dog contained less than 0.2 per cent of the 22.5 mg. of uranyl acetate administered intravenously 3 hours previously.

*Deposition in Soft Tissues*—Within 45 minutes after administration, all of the soft tissues<sup>2</sup> (excepting kidney) contained *in toto* as much as one-fifth

<sup>2</sup> Tissues analyzed separately included bladder, spleen, liver, gonads, heart, lungs, stomach, intestines, skin and hair, and leg muscle. All other non-calcified structures were pooled and analyzed as "soft carcass."

of the uranium administered. At this time there were considerable quantities of uranium in the blood. Later, concurrently with the diminishing blood concentration, the soft tissue content decreased to a very low level (0.2 to 0.3  $\gamma$  per gm) and remained low throughout the 40 day time interval. These results are presented graphically in Fig. 4.

*Deposition in Bone*—The greatest concentration in bone (20 to 30 per cent of the dose) was noted in 2.5 hours after injection. These data are pre-

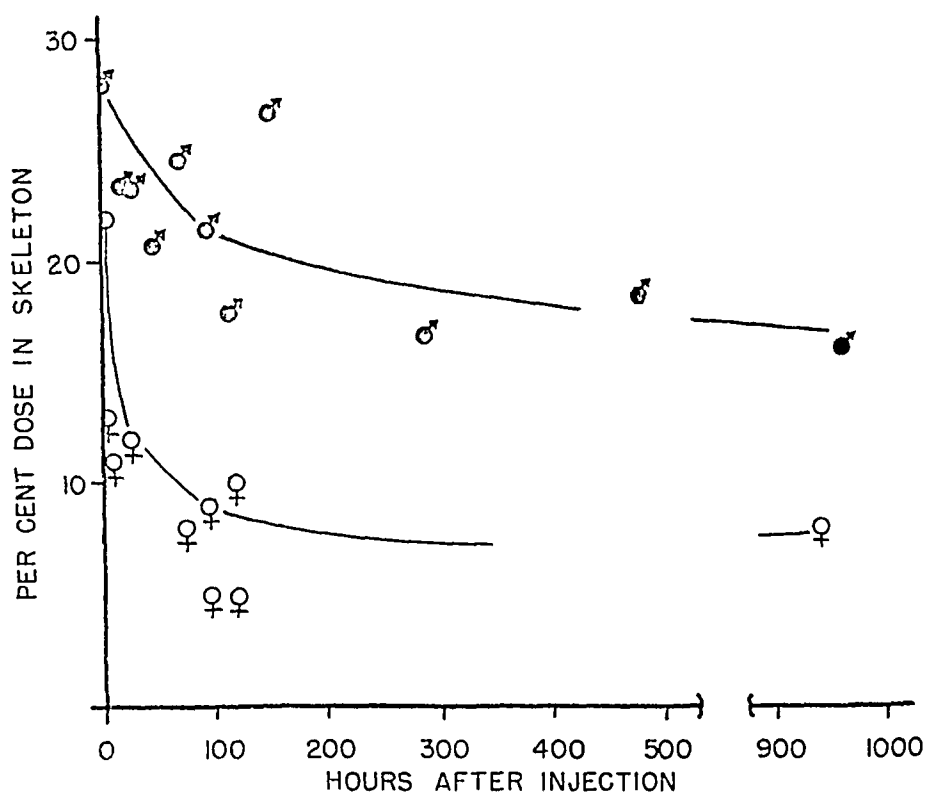


FIG. 5 Data showing a marked sex difference in the deposition of uranium in the rat skeleton

sented in Fig. 5. Though gradually decreasing thereafter, the quantity found in the skeleton at 40 days accounted for 90 per cent of that remaining in the animal. A consistently greater amount was stored in the bones of males than of females.

In view of the fact that liver, spleen, and all soft tissues contained very low concentrations (less than 0.5  $\gamma$  per gm), there was little doubt that the uranium found in the skeleton resided in the bone substance and *not* the bone marrow. To confirm this, a femur was removed from a rabbit injected

intravenously with uranyl acetate, separated into four portions, and analyzed with the following results

	Sample weight gm	Total U content %
Femur end	5.35	91.2
Marrow from femur end	0.34	0.33
Femur shaft	2.07	25.2
Marrow from femur shaft	1.05	0.66

These data, in spite of the possibility of cross-contamination, clearly showed that marrow does not store significant amounts of injected uranium. As in the case of many other minerals, the end of the femurs, including epiphysis and metaphysis, showed a greater uptake (17  $\gamma$  per gm) than did the shaft (12.7  $\gamma$  per gm).

#### DISCUSSION

The rapidity with which uranium left the circulation and appeared in the urine indicates that uranium exists in the animal for the most part in a soluble, diffusible state. Although uranium appeared in the soft tissues shortly after injection, if it penetrated the cells, it was not fixed there to any appreciable extent.

Bone, on the other hand, showed a remarkable affinity for uranium. It is interesting that a consistently greater amount was stored in the bones of males than of females. This suggested that some physiological factor related to sex is involved in bone deposition. However, subsequent studies<sup>2</sup> revealed that the factor in question was age rather than sex. In the experiments reported here, all rats studied weighed  $200 \pm 5$  gm. At this weight, the average male rat of our colony is 9 weeks of age, the average female 16 weeks. The males, therefore, because of a greater rate of bone growth, deposited greater quantities of uranium in the skeleton.

It appeared that the urinary system (kidney and urine collectively) was in competition with the skeletal system for the injected uranium. For example, 100 hours after injection the males showed approximately 20 per cent in the skeleton, 66 per cent in the kidney and urine, the females, 8 per cent in the skeleton, 80 per cent in the kidney and urine. The fact that young animals deposit more uranium in the skeleton,<sup>3</sup> leaving a smaller proportion of the dose to attack the kidney, may account in a large measure for the "resistance" of young animals to the nephrotoxic effects of injected uranium, a fact reported by MacNider (14) and confirmed in this laboratory.

From Table VI it is evident that variations in acid-base balance do not materially alter the distribution between the skeleton and the urinary sys-

<sup>2</sup> Neuman, M. W., Neuman, W. F., Main, E., and Mulryan, E., unpublished results.

tem Rather, it determines to what extent the uranium which reaches the kidney is initially fixed in that tissue Alkali infusion kept kidney fixation below 1 per cent of the dose Ammonium chloride feeding, on the other hand, increased the deposition in the kidney to as much as 72 per cent This finding provides a rational basis for the fact that alkali administration diminishes the nephrotoxic effects of injected uranium

#### SUMMARY

Experiments on the distribution and excretion of injected uranium were conducted on rats, rabbits, and cats

Roughly two-thirds of the dose was excreted rapidly via the urine As much as one-fifth was found in the skeleton from which it was mobilized slowly The kidney initially fixed as much as one-fifth of the dose, but the concentration fell rapidly to a very low value in 40 days All other soft tissues (including blood) contained negligible quantities

Alkali administration reduced the amount deposited in kidney, increasing proportionately the quantity found in urine Ammonium chloride feeding exerted an effect opposed to that of alkali administration

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# MERCAPTALS AND MERCAPTOLES OF CYSTEINE

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Our earlier synthesis of djenkolic acid through the condensation of methylene chloride and cysteine (1) in liquid ammonia did not appear to lend itself to the preparation of certain related mercaptals and mercaptoles which we desired. However, the reaction which we have recently described (2) between formaldehyde and cysteine in a strongly acid solution to give djenkolic acid appeared to offer attractive possibilities in this direction. We have now extended this reaction to acetaldehyde, propionaldehyde, and benzaldehyde. When these aldehydes were added to a concentrated solution of cysteine in 6 N hydrochloric acid, the reaction proceeded vigorously and was completed in a few minutes. Some of the products formed insoluble monohydrochlorides and were best purified by recrystallization of this derivative.

We have also found that when acetone and cyclohexanone were caused to react with cysteine under the conditions used for the formation of mercaptals, the corresponding cysteine mercaptoles were obtained.

## EXPERIMENTAL

*L-Cysteine Mercaptal of Acetaldehyde*—To a solution of 1.0 gm. of *L*-cysteine hydrochloride in 2 cc. of 6 N HCl was added 0.65 cc. (0.5 mole) of acetaldehyde, and the solution was allowed to stand at room temperature. After a few seconds the solution became hot and the monohydrochloride of the mercaptal began to crystallize. The mixture was cooled in an ice bath and the solid was collected on a sintered glass filter, washed with cold 6 N HCl, and dried. The yield was 0.70 gm. (65 per cent of the theoretical amount). It was recrystallized several times from 50 per cent ethanol,  $[\alpha]_D^{20} = -15.0^\circ$  for a 1 per cent solution in 1 N HCl, m.p. 205–230° (with decomposition).<sup>1</sup> The compound gave a negative test for the sulfhydryl and disulfide groups and was not oxidized by iodate.

$C_4H_{11}O_4N_2S_2Cl$	Calculated	C 31.52, H 5.59, N 9.20, S 21.02
304.5	Found	" 31.98, " 5.86, " 9.25, " 21.20

*L-Cysteine Mercaptal of Propionaldehyde*—The preceding experiment was repeated with 0.24 cc. (0.5 mole) of propionaldehyde. The product

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<sup>1</sup> All melting points are corrected micro melting points.



was obtained and purified in the same manner as the corresponding compound from acetaldehyde. The yield was 0.90 gm (88 per cent of the theoretical amount),  $[\alpha]_D^{20} = +16.0^\circ$  for a 1 per cent solution in 1 N HCl, m p  $220-222^\circ$  (with decomposition)

$C_7H_{10}O_4N_2S_2Cl$	Calculated	C 33.91, H 6.39, N 8.79, S 20.09
318.5	Found	" 34.41, " 6.62, " 8.65, " 20.43

*L-Cysteine Mercaptal of Benzaldehyde*—To a solution of 0.5 gm of L-cysteine hydrochloride in 1 cc of 6 N HCl was added 0.33 cc (0.5 mole) of benzaldehyde and the mixture was shaken at intervals. It became warm and the benzaldehyde gradually dissolved. After standing at room temperature for an hour, the clear solution was diluted with an equal volume of water, the excess benzaldehyde was extracted with ether, and the solution was made neutral to litmus by the addition of 6 N NaOH. The resulting gelatinous precipitate dissolved when the mixture was heated, but reprecipitated as a gel when the solution was cooled. The compound crystallized as small white rosettes after standing overnight at room temperature. The yield was 0.45 gm (78 per cent of the theoretical amount). The compound was twice recrystallized from water, each time precipitating as a gel which slowly crystallized as small rosettes. The compound gave a negative test for chloride and a negative test for the sulphydryl and disulfide groups,  $[\alpha]_D^{20} = +35.0^\circ$  for a 1 per cent solution in 1 N HCl, m p  $200-220^\circ$  (with decomposition)

$C_{13}H_{18}O_4N_2S_2$	Calculated	C 47.27, H 4.58, N 8.48, S 19.39
330	Found	" 47.48, " 5.80, " 8.59, " 19.55

*L-Cysteine Mercaptol of Acetone*—To a solution of 2.5 gm of L-cysteine hydrochloride in 5 cc of 6 N HCl were added 1.1 gm (1 mole) of acetone. After the solution had stood at room temperature overnight, the mercaptol monohydrochloride had crystallized. The precipitate was collected, washed with cold 6 N HCl, and dried. The yield was 2.3 gm (83 per cent of the theoretical amount). After several recrystallizations from water the compound continued to give a positive test for the sulphydryl group when treated with sodium nitroprusside.

$C_9H_{12}O_4N_2S_2Cl$	Calculated	C 33.91, H 6.39, N 8.79, S 20.09
318.5	Found	" 34.23, " 6.21, " 8.90, " 20.25

*L-Cysteine Mercaptol of Cyclohexanone*—To a solution of 1.0 gm of L-cysteine hydrochloride in 2 cc of 6 N HCl was added 0.34 cc (0.5 mole) of cyclohexanone. The solution was heated to  $70^\circ$  and was allowed to cool and to stand at room temperature for several days. It was then cooled in an ice bath and was made neutral to litmus by the addition of 6 N NaOH. The mercaptol precipitated as a bulky solid which was collected on a

filter, washed with a small amount of cold water, and dried. The yield was 0.20 gm (20 per cent of the theoretical amount). After two recrystallizations from aqueous ethanol the compound gave a negative test for the sulfhydryl and disulfide groups. The rotation was too small to be measurable for a 1 per cent solution in 1% HCl, m.p. 245–247° (with decomposition).

$C_{12}H_{12}O_4N_2S_2$	Calculated	C 44.58, H 6.81, N 8.68, S 19.82
323	Found	" 44.68, " 6.93, " 8.37, " 20.07

#### SUMMARY

Cysteine will condense with simple aldehydes in strongly acid solution to yield cysteine mercaptals. The cysteine mercaptols of acetone and cyclohexanone were prepared under the same conditions.

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# THE REQUIREMENTS OF THE FATTY ACID OXIDASE COMPLEX OF RAT LIVER\*

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In an earlier paper it was shown that suspensions of the washed particulate portion of homogenized rat liver readily oxidized saturated fatty acids in the presence of adenosine triphosphate (ATP),  $Mg^{++}$ , and inorganic phosphate (1). The information outlined in that paper allowed a quantitative analysis of the products of enzymatic fatty acid oxidation to be made (2), since such enzyme preparations were essentially free of endogenous respiratory activity. The oxidation of *n*-octanoate in these suspensions was found to proceed according to the equation,  $C_7H_{15}COOH + 3O_2 \rightarrow 2CH_3COCH_2COOH + 2H_2O$ . However, when the oxidation was allowed to take place in the presence of oxalacetate, the yield of acetoacetate diminished and citrate accumulated, implicating the Krebs tricarboxylic acid cycle in fatty acid oxidation in these enzyme preparations. The latter study also yielded some information as to certain features of the mechanism of fatty acid oxidation, supplementing the isotope studies of Weinhouse and his colleagues (3, 4) and Buchanan *et al* (5). The gross mechanism of oxidation in the liver of the rat, at least, appears to involve the successive oxidative removal of 2-carbon units from the fatty acid. These units (which are not necessarily identical) may recondense with each other to form acetoacetate or they may react with oxalacetate to form tricarboxylic acids and thus enter into the Krebs cycle.

With this broad outline of the gross mechanism of enzymatic fatty acid oxidation as a guide, attention in this laboratory was again turned to the study of the properties of the fatty acid oxidase complex with the end in mind of dissociating and characterizing separate reaction steps. In the course of attempts to isolate individual reactions of the highly integrated complex of enzymes by variations in the method of preparation, it was found that the enzyme system required several factors in addition to ATP and  $Mg^{++}$ . In this paper experiments are described which led to recognition of some additional cofactors in enzymatic fatty acid oxidation and certain properties of the enzyme complex.

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## EXPERIMENTAL

*Analytical Methods*—Acetoacetate was determined colorimetrically by a modification of the method of Greenberg and Lester (6) and citrate by the method of Speck, Moulder, and Evans (7). Octanoate, the fatty acid used as substrate throughout this investigation, was estimated in copper-lime filtrates by a specific method devised in this laboratory (8).

Oxygen uptake was measured in Warburg vessels equipped with alkali and filter paper roll. All manometric measurements were made in air at 30°. In the manometric experiments all components of the reaction medium were present in the main compartment of the Warburg vessels, which were placed in cracked ice. The ice-cold enzyme was added last, and the flasks then placed on manometers and brought to temperature equilibrium in the bath. A 5 to 8 minute temperature equilibration period preceded manometric measurements.

Inorganic and total phosphorus was measured by the Fiske and Subbarow method adapted to photoelectric measurement. Acid-soluble P, phospholipid P, and total nucleic acid P were separated and determined by the method of Schneider (9). Differentiation between pentose- and desoxypentosenucleic acid P was made by the method of Schmidt and Thannhauser (10).

The radioactivity measurements were made on thin layers of aqueous solutions with a standard Geiger-Müller counting tube and conventional scaling apparatus.

*Preparative*—Adenine nucleotides were prepared as mentioned in a previous paper (1). The diphosphopyridine nucleotide (DPN) used was a product of the Schwarz Laboratories, stated to be 60 per cent pure. Spectrophotometric determination (11) of the purity yielded a value of 51 per cent. The radioactive phosphorus was obtained in the form of  $\text{Na}_2\text{HP}^{32}\text{O}_4$ , essentially carrier-free, through the United States Atomic Energy Commission.

Two types of enzyme suspension will be referred to throughout this paper, the *saline* suspension and the *water* suspension, and since the manner of preparation of the enzymes is of considerable importance in their requirements for activity, this will be described in detail.

The basic material used in the two types of enzyme preparation is the washed particulate matter of rat liver prepared by a slight modification of the method previously used (1). Iced rat liver, freshly removed from the exsanguinated animal, was homogenized with 2 volumes of ice-cold 0.13 M KCl-0.013 M sodium phosphate buffer, pH 7.8. The homogenate was strained through gauze and diluted with an equal volume of cold KCl-buffer solution. The mixture was centrifuged at 2200*g* in an angle head centrifuge in the cold for 3 minutes. The supernatant was discarded. Fresh, cold

KCl buffer was added to the residue to equal the original volume of diluted homogenate. The residue was resuspended by shaking and again centrifuged in the cold (2 minutes, 2200g). The supernatant was decanted and the residue again taken up in the same volume of KCl-buffer and centrifuged. After decanting the supernatant from the last washing, the residue remaining was used to prepare the two types of enzyme suspension. For the *water* suspension the residue was taken up in cold distilled  $H_2O$  to the volume of the original diluted homogenate. For the *saline* suspension the residue was taken up in cold 0.13 M KCl-0.013 M phosphate to the volume of the original diluted homogenate. The *saline* suspension is enzymatically equivalent to the preparation described in a previous paper (1).

*Effect of Suspending Enzyme in Water on Activity Requirements*—In an earlier paper it was shown that *saline* suspensions of saline-washed particulate matter of rat liver readily oxidized octanoate when supplemented with ATP,  $Mg^{++}$ , and phosphate buffer (1). It was also found that if water was used instead of saline for preparation or suspension of the insoluble particles no enzymatic activity could be obtained. Potter has independently confirmed these findings (12). He ascribed the dependence of enzyme activity in the presence of saline in approximately isotonic concentrations to the possibility that the oxidation was taking place only in intact cells, a view which he has since abandoned (13). He also found that when the preparations made by suspending the saline-washed enzyme in water were tested in the presence of extra salt in the Warburg vessel activity could be restored in some preparations but the effect could not be consistently obtained (14).

We have been able to confirm the restoring effect of salt on the *water* suspension of enzyme. However, the restoration of activity was found to be quite erratic, some preparations of enzyme remaining completely inactive.

It was soon found, however, that if low concentrations of certain intermediates of the Krebs tricarboxylic acid cycle were present in the reaction medium in addition to KCl, ATP,  $Mg^{++}$ , and phosphate, uniform restoration of activity could be obtained. Furthermore, under these conditions a requirement of cytochrome *c* became much more strongly evident than was the case with the *saline* suspensions of enzyme previously studied (see also Potter (12)). In Table I are shown the data collected from experiments designed to compare the requirements for activity of the *saline* suspension of enzyme and the *water* suspension of enzyme. The *saline* suspension of the enzyme does not require the addition of KCl, malate, or cytochrome *c* for strong activity, these substances do not accelerate the oxidation greatly when added to the system. However, in the *water* suspension of enzyme the absolute nature of the requirement for neutral salt and malate is strikingly evident, as well as the substantial requirement for the presence of cytochrome *c*. The criteria of fatty acid oxidase activity were measure-

ments of oxygen uptake, octanoate disappearance, and acetoacetate formation

Treatment of the saline-washed particulate matter of rat liver with water, then, reveals that the enzymatic oxidation of octanoate to acetoacetate requires the presence of ATP,  $Mg^{++}$ , a neutral salt such as KCl, a small amount of malate, and cytochrome *c*. If the saline-washed particles are

TABLE I

*Comparison of Requirements of Saline and Water Suspensions of Enzyme*

In the *saline* suspension test, the Warburg vessels contained 1.7 ml of *saline* suspension (added last), 0.25 ml of  $MgSO_4$  (0.005 M),\* 0.25 ml of KCl (0.05 M), 0.50 ml of phosphate buffer, pH 7.4 (0.01 M), 0.50 ml of ATP (0.0005 M), 0.25 ml of sodium octanoate (0.001 M, total 5.0 micromoles), 0.50 ml of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.25 ml of sodium *l*-malate (0.0003 M), and water to make 5.0 ml. When the components were omitted, an equal volume of water was substituted. Time, 40 minutes. In the *water* suspension test, the system was arranged exactly as in the *saline* suspension test. Time, 55 minutes.

		Oxygen uptake	Octanoate removed	Acetoacetate formed
		micromoles	micromoles	micromoles
Saline suspension	Complete system	16.7	4.8	9.2
	Octanoate omitted	2.0		0.3
	Malate omitted	14.0	4.9	10.4
	KCl omitted	14.8	4.8	9.1
	$MgSO_4$ omitted	4.3	1.1	3.4
	ATP omitted	0.6	0.4	0.3
	Cytochrome <i>c</i> omitted	14.2	4.4	8.1
Water suspension	Complete system	13.6	4.5	7.9
	Octanoate omitted	3.1		0.1
	Malate omitted	1.0	0.1	0.2
	KCl omitted	1.7	0.1	0.0
	$MgSO_4$ omitted	4.3	1.6	3.2
	ATP omitted	0.2	0.0	0.2
	Cytochrome <i>c</i> omitted	3.6	2.0	2.0

\* The figures in parentheses refer to final concentration of each component in the complete reaction medium. This notation is used in all the tables in this paper.

suspended in saline, however, they show an absolute requirement for only ATP and  $Mg^{++}$ .

The effects and possible functions of some of the components required for fatty acid oxidase activity will now be considered.

*Requirement of Adenine Nucleotides*—Previous work indicated that in the *saline* suspension of washed liver particles ATP was required for fatty acid oxidation, whereas adenosine diphosphate (ADP) and muscle adenylic acid were ineffective (1). However, it has since been found that under certain

conditions the three nucleotides are equally effective, both in the *saline* suspension and in the *water* suspension. In the earlier study the enzyme was incubated with all components of the system except substrate for some 5 minutes (temperature equilibration period), followed by addition of substrate from the side arm. Under these conditions only ATP was found effective. However, if *all* components including fatty acid substrate are initially present in the main compartment of the chilled Warburg vessel, ice-cold enzyme being added last, and the contents then incubated, all three nucleotides are equally effective in activating the oxidation. Study of the rates of dephosphorylation of the nucleotides by the enzyme revealed that all three nucleotides undergo rapid dephosphorylation. In the 5 minute period of incubation before substrate was added (in the earlier experiments)

TABLE II  
*Requirement of Adenine Nucleotides*

The main compartment of the Warburg vessels contained 0.90 ml of *water* suspension of enzyme, 0.15 ml of  $\text{MgSO}_4$  (0.005 M), 0.15 ml of KCl (0.05 M), 0.30 ml of nucleotide, or adenosine, or adenine, 0.30 ml of phosphate buffer, pH 7.4 (0.01 M), 0.30 ml of cytochrome *c* ( $10^{-6}$  M), 0.15 ml of *l*-malate (0.0005 M), 0.15 ml of sodium octanoate (0.001 M), and water to make 3.0 ml. Time, 40 minutes

	Oxygen uptake	Acetoacetate formed
	<i>micromoles</i>	<i>micromoles</i>
0.001 M ATP	8.1	2.5
0.001 " ADP	8.4	3.0
0.001 " muscle adenylic acid	9.3	2.6
0.001 " yeast " "	1.4	0.0
0.001 " adenosine	0.4	0.1
0.001 " adenine	0.5	0.1
0.001 " DPN (51% pure)	8.9	2.4
0.0001 " " (51% " )	3.2	0.7
No addition	0.7	0.0

extensive dephosphorylation and deamination of the nucleotides were obviously taking place. However, since ATP has three phosphate groups which must be consecutively removed by phosphatase action to form adenosine, which is inactive, it is possible that the apparently specific effectiveness of ATP in the earlier experiments was due to sparing of some of the nucleotide by the presence of the additional phosphate groups. After fatty acid oxidation has once begun, phosphorylations coupled to the oxidation continually regenerate ATP or ADP from adenylic acid, as experiments below will show.

In Table II are shown data obtained with the *water* suspension of enzyme, indicating the effectiveness of the three nucleotides and the ineffectiveness of yeast adenylic acid, adenosine, and adenine under the same conditions.



In the course of these experiments it was found that preparations of DPN of approximately 50 per cent purity can replace the adenine nucleotides in the system. Whether this is due to the presence of adenine nucleotides as impurities in the DPN preparations or to the enzymatic scission of DPN to form an adenine mononucleotide is not clear. This effect of DPN may be related to the occasional effectiveness of DPN in restoring activity of aged fatty acid oxidase preparations (1).

Although all three adenine mononucleotides are effective in activating the oxidation of octanoate, it may not be safely concluded that adenylic acid, as the least common denominator, is the immediately required nucleotide, since data reported below show that the nucleotides undergo active aerobic phosphorylation. It is quite possible that a mixture of all three nucleotides may be present during the oxidation following the addition of any one nucleotide, as a resultant of phosphorylation and dephosphorylation reactions.

Since earlier work pointed to ATP as the active nucleotide, a tentative hypothesis had been set up at that time concerning the mechanism of action of ATP which involved an obligatory phosphorylation of fatty acid by the ATP prior to oxidation (15, 16). This hypothesis gained some experimental support when it was found that synthetic acyl phosphates of higher fatty acids (17) readily donated phosphate enzymatically to adenylic acid in crude homogenates of rat liver.<sup>1</sup> Subsequently, the development of the washed enzyme suspension, free of endogenous activity (1), allowed a more critical test of the synthetic acyl phosphates as intermediates in fatty acid oxidation. It was found that the washed liver suspensions, in contrast to the crude liver homogenate, were not capable of catalyzing the transphosphorylation reaction observed in the crude homogenate. Furthermore, the synthetic acyl phosphates show no special activities in either the *saline* suspension or *water* suspension of the washed enzyme not shown by free fatty acid salts. The transphosphorylation reaction observed in crude liver homogenates, therefore, appears not to be concerned in fatty acid oxidation. It may, however, be concerned in other metabolic reactions of fatty acids.

*Requirement of Inorganic Phosphate*—In Table III are shown results of an experiment demonstrating that the presence of inorganic phosphate is required for fatty acid oxidation. Since the reaction medium was ordinarily buffered with inorganic phosphate, it was necessary to substitute another buffer. Tris(hydroxymethyl)aminomethane-HCl buffer (18) proved to be ideal for the system. It can be seen that omission of inorganic phosphate results in greatly decreased activity. The small amount of inorganic phosphate already present in the enzyme preparations probably is responsible for this minimal activity. Raising the concentration of inorganic phos-

<sup>1</sup> Lehninger, A. L., unpublished experiments.

phate causes concomitant increases in the rate of oxidation until a plateau is reached at a concentration of approximately 0.0005 M inorganic phosphate. Obviously, the system is capable of functioning maximally with rather low concentrations of inorganic phosphate.

The requirement of inorganic phosphate for activity of the fatty acid oxidase system is probably related to the ability of the system to cause esterification of inorganic phosphate coupled to the oxidation of fatty acid. In Table IV are shown supporting data. It can be seen that in the complete system the oxidation of fatty acid maintains the level of acid-labile esterified phosphate (P liberated by 7 minutes hydrolysis at 100° in 1 N

TABLE III  
*Inorganic Phosphate Requirement*

The Warburg vessels contained 0.90 ml of water suspension of enzyme, 0.15 ml of  $\text{MgSO}_4$  (0.005 M), 0.15 ml of KCl (0.05 M), 0.30 ml of cytochrome *c* ( $10^{-5}$  M), 0.30 ml of sodium adenylate (0.001 M), 0.15 ml of octanoate (0.001 M), 0.30 ml of tris(hydroxymethyl)aminomethane HCl buffer, pH 7.5, 0.15 ml of malate (0.0005 M), concentrations of inorganic phosphate indicated below, and water to make 3.0 ml. Time, 30 minutes.

Inorganic phosphate		O <sub>2</sub> uptake	Acetoacetate formed
Added	Present*		
M	M	micromoles	micromoles
0	0.0002	1.3	0.3
0.0001	0.0003	2.7	1.4
0.0002	0.0004	5.9	2.5
0.0003	0.0005	7.1	3.8
0.0004	0.0006	7.2	3.6
0.001	0.0012	7.6	3.9
0.010	0.010	7.4	3.8

\* The enzyme preparation contained sufficient inorganic phosphate to give a concentration of about 0.0002 M.

$\text{H}_2\text{SO}_4$ ) When fatty acid is omitted, there is some maintenance of the esterified phosphate owing to the oxidation of the small amounts of malate present. When both substrates are omitted, there is no oxidation and no significant maintenance of esterified phosphate. The maintenance of esterified phosphate in the complete system is probably due to the fact that the rate of esterification of inorganic phosphate coupled to the oxidation of fatty acid approaches the rate of dephosphorylation of phosphate esters by phosphatases, the result being maintenance of the level of esterified phosphate. In the absence of oxidations no esterification takes place and the phosphatases quickly dephosphorylate the adenine nucleotides and other

phosphate esters present. Several attempts have been made to demonstrate a *net* synthesis of newly esterified phosphate by using various phosphate acceptors such as creatine, glucose, glucose-6-phosphate, etc., to trap esterified phosphate. However, the necessary transphosphorylases appear not to be present in these enzyme suspensions.

In order to demonstrate unequivocally that the maintenance of esterified phosphate shown in Table IV is actually the resultant of the oxidation-coupled synthesis of new phosphate linkages and phosphatase action, we have allowed the oxidation of octanoate to take place in a medium containing inorganic phosphate labeled with  $P^{32}$ . After completion of the incubation, trichloroacetic acid filtrates were prepared and the radioactivity of

TABLE IV

*Maintenance of Level of Esterified Phosphate by Octanoate Oxidation*

The Warburg vessels contained 0.90 ml of water suspension of enzyme, 0.15 ml of  $MgSO_4$  (0.005 M), 0.15 ml of KCl (0.05 M), 0.60 ml ATP (= 227  $\gamma$  of 7 minute-hydrolyzable P), 0.30 ml of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.30 ml of tris(hydroxymethyl)-aminomethane-HCl buffer, pH 7.4 (0.01 M), 0.15 ml of octanoate (0.001 M), 0.15 ml of *l*-malate (0.0005 M), and  $H_2O$  to make 3.0 ml. When components were omitted,  $H_2O$  was substituted. The side arm contained 0.3 ml of 3 N HCl, tipped to stop reaction at specified times.

	Time	O uptake	Acetoacetate formed	Inorganic P	7 min hydrolyzable P
	min	micromoles	micromoles	$\gamma$	$\gamma$
Complete system	0			113	256
" "	25	6.5	3.0	148	248
Octanoate omitted	25	1.2	0.1	278	105
<i>l</i> -Malate "	25	1.1	0.3	423	65
Both octanoate and <i>l</i> -malate omitted	25	0.3	0.0	445	35

the esterified fraction of the acid-soluble phosphorus was measured after removal of inorganic  $P^{32}$  by dilution with carrier phosphate and separation with magnesia mixture. The results, shown in Table V, clearly demonstrate substantial incorporation of inorganic  $P^{32}$  into the esterified fraction coupled to fatty acid oxidation. When octanoate was omitted, some esterification took place owing to the oxidation of the malate present. When both octanoate and malate were omitted, very little incorporation occurred. These experiments, therefore, demonstrate that at least part of the free energy released during fatty acid oxidation is recovered by coupled synthesis of new phosphate bonds.

*Malate Requirement*—Since the addition of small amounts of certain intermediates of the Krebs cycle to the reaction medium was found to be neces-

sary for complete restoration of the activity of the *water* suspension of enzyme, this effect was studied from the standpoint of specificity. As has been mentioned before, not all preparations require the addition of such a compound for fatty acid oxidase activity, presumably because small but sufficient amounts of such substances are already present in some of the enzyme preparations. In order to study this effect quantitatively, it was necessary to use only enzyme preparations which showed no fatty acid oxidase activity unless the activator of the Krebs cycle was present. Such preparations were consistently obtained by the simple expedient of making the enzyme preparation with only one-half of the tissue concentration ordinarily used so that the end-product, the *water* suspension of enzyme, con-

TABLE V

*Incorporation of Inorganic Phosphate Labeled with  $P^{32}$  into Esterified Phosphate Fraction Coupled to Fatty Acid Oxidation*

The main compartment of the Warburg vessels contained 0.60 ml of diluted *water* suspension of enzyme, 0.10 ml of KCl (0.05 M), 0.10 ml of  $MgSO_4$  (0.005 M), 0.20 ml of tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.5 (0.01 M), 0.40 ml of ATP (0.001 M), 0.20 ml of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.10 ml of malate (0.0005 M), 0.10 ml of octanoate (0.001 M), 0.10 ml of inorganic phosphate containing  $P^{32}$  (363,300 counts per minute, total), and  $H_2O$  to make 2.0 ml. Incubated at  $30^\circ$  for 8 minutes, taps closed and manometric measurements made for the following 17 minutes.

	O uptake	Inorganic P	Esterified P	Specific activity of esterified P
	micromoles	$\gamma$	$\gamma$	counts per min per $\gamma$ esterified P
Complete system (0 time)		120	194	12
"	3.2	166	157	1010
Octanoate omitted	0.3	223	93	590
" and malate omitted	0.2	246	54	167

tained only about one-half the usual enzyme concentration and was somewhat more thoroughly washed. These preparations were used in the experiments reported in Table VI. In Experiments I and II a variety of compounds were tested for their ability to activate octanoate oxidation, all other known requirements being supplied at optimal concentration. The criteria used were oxygen uptake and octanoate utilization. In the absence of added activating compound, no octanoate oxidation took place. The compounds found to be effective in activating the oxidation were *cis*-aconitate, citrate, malate, oxalacetate,  $\alpha$ -ketoglutarate, succinate, and fumarate. However, lactate, pyruvate, *dl*- $\beta$ -hydroxybutyrate, acetate, ascorbate, and *dl*-phosphomalate were found to be inactive when tested at the same concentration (0.0005 M). Acetaldehyde showed a slight activat-

ing effect Octanoate oxidation, therefore, requires the presence of some intermediate of the Krebs tricarboxylic acid cycle other than pyruvate

Further experiments disclosed that the rate of oxidation of isocitrate to  $\alpha$ -ketoglutarate is the slowest oxidative reaction of the Krebs cycle in these diluted enzyme preparations Citrate and *cis*-aconitate when tested for their activating effect on octanoate oxidation show a considerable lag period

TABLE VI

*Requirement of Intermediates of Krebs Cycle*

The main compartment of the Warburg vessels contained 1.5 ml of dilute water suspension (see the text), 0.25 ml of  $\text{MgSO}_4$  (0.005 M), 0.25 ml of KCl (0.05 M), 0.50 ml of ATP (0.0006 M), 0.50 ml of phosphate buffer, pH 7.4 (0.01 M), 0.50 ml of cytochrome *c* ( $1 \times 10^{-6}$  M), 0.25 ml of octanoate (0.001 M), 0.25 ml of activating substrate (0.0005 M), and water to make 5.0 ml In Experiment III, 0.01 M malonate was also present Time, 60 minutes in each experiment

Experiment No	Activator	O <sub>2</sub> uptake	Octanoate utilized
		micromoles	micromoles
I	None	0.1	0.2
	<i>l</i> -Malate	18.8	4.9
	Oxalacetate	17.6	4.9
	<i>dl</i> - $\beta$ -Hydroxybutyrate	1.7	0.2
	Pyruvate	2.5	0.3
	Acetaldehyde	4.0	0.6
	<i>dl</i> -Phosphomalate	1.0	0.1
	Acetate	1.2	0.2
II	None	0.9	0.4
	<i>cis</i> -Aconitate	8.2	2.4
	Citrate	8.0	2.7
	$\alpha$ -Ketoglutarate	10.1	3.7
	Succinate	10.2	3.9
	Fumarate	10.0	3.9
	Lactate	1.2	0.6
	Ascorbate	1.1	0.3
III	None	0.0	0.2
	$\alpha$ -Ketoglutarate	1.3	0.4
	Succinate	0.1	0.1
	<i>l</i> -Malate	4.2	1.2
	Oxalacetate	4.4	1.5

before extensive removal of octanoate occurs No lag period is observed with  $\alpha$ -ketoglutarate, succinate, malate, or oxalacetate under the same conditions Tentatively, the presence of citrate or the oxidation of citrate to  $\alpha$ -ketoglutarate may be excluded as the activating factor

Further localization of the activating reaction was achieved by the use of malonate as an inhibitor of succinic dehydrogenase (Table VI, Experiment

III) Although malonate also inhibited fatty acid oxidation strongly in these experiments,<sup>2</sup> it was found that citrate,  $\alpha$ -ketoglutarate, and succinate failed to activate octanoate oxidation in the presence of malonate, whereas malate and oxalacetate were still capable of activating the oxidation. The activating effect of the intermediates of the Krebs cycle may therefore be tentatively localized to some effect of the presence of malate or oxalacetate.

Previous work has shown that intermediates of the Krebs cycle, when added to the *saline* suspension of enzyme, cause a decrease in yield of acetoacetate from the theoretical 2 moles per mole of octanoate oxidized, some of the fatty acid carbon being diverted into the formation of citrate via a 2-carbon intermediate (2). The present study indicates that malate or oxalacetate is required in the water-treated enzyme for the oxidation of octanoate to acetoacetate. The latter finding implies that malate actually has two distinct functions in the oxidase system, one to activate the oxidation of octanoate to acetoacetate (or the 2-carbon intermediate), and the other to react with 2-carbon units to form citrate and thus cause fatty acid oxidation products to enter the Krebs cycle. The results of the experiment presented in Table VII illustrate the dual function of malate in the fatty acid oxidase system. In this experiment the *water* suspension of enzyme was allowed to act upon octanoate in the presence of varying concentrations of malate. Measurements of oxygen uptake, octanoate utilization, acetoacetate formation, and citrate formation were made. In the absence of added malate there was no oxidation of octanoate. With 0.0001 M malate suboptimal oxidation of octanoate occurred. At 0.0005 M malate concentration, maximal octanoate disappearance occurred. Somewhat less than the theoretical yield of 2 moles of acetoacetate was formed per mole of octanoate disappearing. Raising the malate concentration from 0.0005 M to as high as 0.02 M caused no increase in the octanoate utilization, but produced a great decrease in yield of acetoacetate and a great increase in yield of citrate. Therefore, it is clear that a small amount of malate is required to initiate oxidation of the fatty acid, in higher concentrations malate has the additional effect of diverting fatty acid carbon from acetoacetate formation into citrate formation, probably by furnishing a greater supply of oxalacetate for condensation to form tricarboxylic acid.

The amounts of malate required for the primary phase, *i.e.* catalysis of

<sup>2</sup> It has been found that malonate has varying inhibitory effects on the fatty acid oxidase system, depending on the strain of rat used for preparation of the enzyme. Preparations made from livers of Sprague-Dawley rats are not inhibited more than about 25 per cent by 0.01 M malonate. In an earlier paper (2) all experiments were done with this strain in the presence of 0.01 M malonate to minimize endogenous respiration. However, preparations made from livers of other strains of rats are much more sensitive. A heterogeneous stock colony used in this work showed great sensitivity, 0.002 M malonate produced 50 to 75 per cent inhibition of octanoate oxidation.

oxidation of octanoate to the stage of acetoacetate or the hypothetical 2-carbon fragment, are quite low and, compared to the amounts of octanoate utilized, are catalytic in magnitude. For instance, in Table VII, at a malate concentration of 0.0001 M (total malate added, 0.5 micromole) 1.5 micromoles of octanoate disappeared. The addition of 1 molecule of malate, which itself is undergoing continuous oxidative removal, therefore sufficed to cause the disappearance of 3 molecules of octanoate. When malate was omitted, no significant disappearance of octanoate occurred.

Since the *saline* suspension of enzyme does not require the addition of catalytic amounts of malate or oxalacetate, it is possible that the activating intermediates are actually present in the particulate material but are "re-

TABLE VII

*Effect of Malate Concentration on Products of Octanoate Oxidation*

The Warburg vessels contained 1.50 ml of dilute water suspension, 0.25 ml of KCl (0.05 M), 0.25 ml of  $MgSO_4$  (0.005 M), 0.50 ml of ATP (0.0005 M), 0.50 ml of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.25 ml of octanoate (0.001 M = 5 micromoles), 0.25 ml of malate in the concentrations listed, 0.50 ml of phosphate buffer, pH 7.4 (0.01 M), and water to make 5.0 ml. Time, 55 minutes.

Malate concentration	Octanoate	O <sub>2</sub> uptake	Octanoate utilized	Acetoacetate formed	Citrate formed
M		micromoles	micromoles	micromoles	micromoles
0	+	0.0	0.2	0.0	0.3
0.0001	+	2.3	1.5	2.7	0.4
0.0005	—	4.4		0.3	0.6
0.0005	+	10.4	3.0	4.3	1.2
0.002	—	5.8		0.0	1.0
0.002	+	12.1	3.1	3.3	2.8
0.008	+	13.2	3.1	1.8	3.4
0.02	—	6.6		0.1	1.2
0.02	+	14.0	3.1	0.8	5.6

leased" from the particles by treatment with water, causing dilution beyond an active concentration. This explanation may also account for the necessity of adding cytochrome *c* after water treatment, since the *saline* suspension does not require the addition of cytochrome *c* for strong activity. If this particulate material represents some subcellular structure preexisting in the cell, it is possible that subjecting the particles to hypotonic solutions causes a change in permeability of a limiting membrane with loss of solutes from within the structure. Certain intracellular inclusions are known to be sensitive to changes in osmotic pressure (19).

*Effect of Salts*—One of the striking properties of the water suspension of enzyme is the complete dependence on KCl for activity. We have investi-

gated the effect of variations in the activity of the enzyme caused by variation in KCl concentration and also the activity of a variety of other compounds substituted for KCl. In Fig 1 are shown the effects of varying KCl concentrations on the oxidase activity and comparable experiments in which NaCl, sucrose, glycine, and urea were substituted for KCl. The criteria of activity were oxygen uptake and acetoacetate formation. Since a number

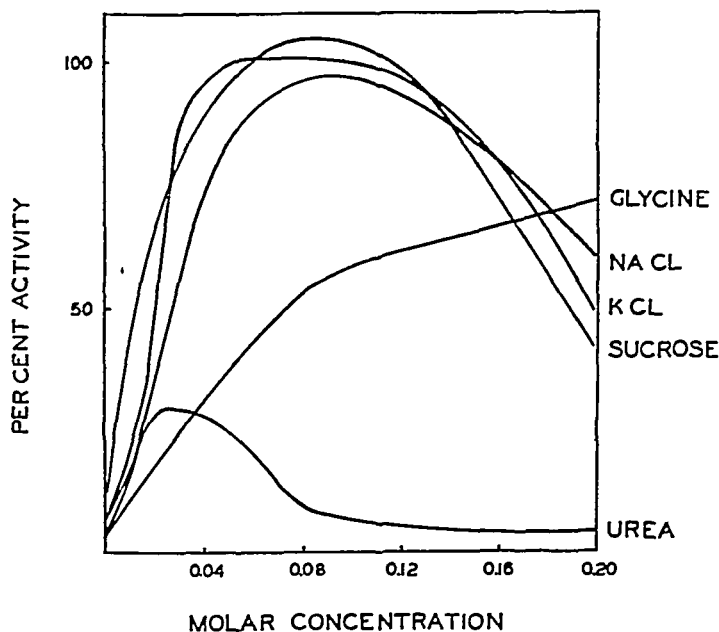


Fig 1 Effect of salts and non-electrolytes on fatty acid oxidase activity. The main compartment of the Warburg vessel contained 0.90 ml of water suspension of enzyme, 0.15 ml of  $MgSO_4$  (0.005 M), 0.60 ml of ATP (0.001 M), 0.30 ml of cytochrome c, 0.30 ml of phosphate buffer, pH 7.4 (0.01 M), 0.15 ml of malate (0.0005 M), 0.15 ml of octanoate (0.001 M), various compounds in final concentrations over the range indicated in the figure, and water to make 3.0 ml. The time varied between 50 and 70 minutes in the different experiments. 100 per cent activity represents acetoacetate formation from octanoate in a system containing 0.05 M KCl.

of different enzyme suspensions were used, a standard system containing 0.05 M KCl was assumed to be 100 per cent active and all activities were expressed in per cent activity of this standard system. In all cases oxygen uptake and acetoacetate formation were parallel.

It can be seen from Fig 1 that KCl was maximally effective in a wide range of concentration from 0.05 M to 0.12 M. Half maximal activation was shown by a concentration of about 0.02 M. Sodium chloride appears to be



equally effective in substituting for KCl. Surprisingly, sucrose showed a nearly identical effect. Glycine, which is not oxidized in these preparations, was also active, although much higher molar concentrations (0.20 M) were necessary to achieve maximal activation. Urea showed some effectiveness at 0.02 M, none at higher concentrations.

Other findings may be summarized. Glucose, fructose, and xylose showed strong activating ability, whereas glycerol, acetone, acetamide, glycogen, and ethylene glycol were inactive. Calcium, magnesium, and barium salts were ineffective. Lithium chloride was as effective as KCl or NaCl. Alanine was somewhat less effective than glycine.

The requirement of salts (or solutes) for oxidative activity is not limited to fatty acid oxidation. The oxidation of malate and the oxidation of pyruvate to acetoacetate by the *water* suspension of enzyme are also completely dependent on the presence of a solute (see Table VIII).

TABLE VIII

*Salt Requirement in Oxidation of Malate and Pyruvate*

The Warburg vessels contained 1.0 ml. of *water* suspension of enzyme,  $MgSO_4$ , KCl, phosphate buffer, cytochrome *c*, and ATP in the same concentrations as in the standard system of Table II plus pyruvate (0.01 M) or malate (0.01 M) (octanoate was not present). When KCl was omitted, water was substituted. Total volume, 3.0 ml. Time, 40 minutes.

Substrate	KCl	O <sub>2</sub> uptake	Acetoacetate formed
		<i>micromoles</i>	<i>micromoles</i>
Pyruvate	+	3.2	3.1
"	—	0.1	0.0
L-Malate	+	4.8	
"	—	0.1	

Potter has presented some evidence that potassium ions have a specific effect in activating the fatty acid oxidase (12). We have found no essential difference between potassium and sodium chloride as activators of the system. However, since KCl was used in the preparation of the enzyme, it appeared that sufficient potassium ions were present in the enzyme to cause maximal activation. In order to test this, a sample of *water* suspension of enzyme was prepared with 0.13 M NaCl-0.013 M phosphate buffer as the homogenizing and washing medium instead of the KCl-phosphate mixture. The washed residue was taken up in water and tested in the presence of 0.05 M NaCl and 0.05 M KCl in separate flasks. Both showed the same activity. Analysis of the enzyme prepared with potassium-free reagents showed that 1.0 ml. of enzyme contained 0.0023 milliequivalent of total potassium. If potassium is a specific requirement of the oxidase, its effect is maximal at concentrations of approximately 0.0007 M in the test system.

During the course of these experiments it was observed that the complete reaction medium, containing the *water* suspension of enzyme and all components required for activity, presented a more opaque or turbid appearance in transmitted light than did the same medium with the activating salt omitted. This observation led to experiments on the *water* suspension of enzyme to determine what physical changes could be brought about by the addition of salts, non-electrolytes, etc. It was found that when KCl was added to the *water* suspension of enzyme to make a concentration of between 0.07 and 0.15 M there was an immediate change from a relatively transparent to an opaque appearance. On centrifuging, a cream-colored precipitate separated sharply, leaving an almost clear supernatant containing hemoglobin. On the other hand, the untreated *water* suspension of enzyme when centrifuged under the same conditions, yielded a small amount of reddish brown precipitate which did not separate sharply from the very turbid supernatant. The effect of KCl on the *water* suspension of enzyme is to bring about a flocculated condition of a component of the suspension. Since the presence of neutral salt is required for fatty acid oxidase activity, this flocculated condition of the enzyme suspension appeared to be associated with the enzyme activity and the dispersed condition (absence of salt) with inactivity.

That this assumption is valid was proved by the following experiment in which the material flocculated from *water* suspension of the enzyme by KCl was separated and tested for enzyme activity. 9 ml. of *water* suspension of enzyme were treated with 1.0 ml. of 1.0 M KCl, allowed to stand for 5 minutes in an ice bath, and then centrifuged in the cold. The slightly turbid, pink supernatant was decanted, leaving a well packed, cream-colored precipitate which was taken up in ice-cold distilled water to make 10.0 ml. Samples of the original *water* suspension, the resuspended KCl-precipitated material, and the supernatant from the latter were assayed for fatty acid oxidase activity. The results are given in Table IX, Experiment I. It is obvious that the material flocculated by KCl contains all of the oxidase activity of the original *water* suspension, the supernatant having no activity. In another experiment, the salt-flocculated enzyme was washed with another portion of 0.1 M KCl and then resuspended in water and tested for activity to determine whether traces of the original supernatant were necessary for activity. This precipitated, washed enzyme was found to be strongly active (Experiment II, Table IX). The data show that this salt-precipitated enzyme also requires neutral salt for activity, as well as other components known to be required by the *water* suspension of washed liver residue.

Analysis of the *water* suspension and the KCl-precipitated enzyme obtained from the *water* suspension for dry weight, total nitrogen, total phosphorus, phospholipid phosphorus, and nucleic acid phosphorus revealed

that the salt-precipitated material contained about 77 per cent of the dry weight, 72 per cent of the total nitrogen, 77 per cent of the total phosphorus, 75 per cent of the phospholipide P, and 95 per cent of the nucleic acid P of the original *water* suspension of enzyme. The salt-precipitated enzyme was nearly devoid of cytochrome *c*, the latter remaining in the supernatant. Analysis (10) of the salt-precipitated enzyme revealed that the nucleic acid present was a mixture of the pentose and desoxypentose types in about equal proportions.

TABLE IX

*Fatty Acid Oxidase Activity of Material Precipitated by KCl from Water Suspension*

The main compartment of the Warburg vessels contained 0.90 ml. of enzyme suspension as indicated below, 0.15 ml. of  $\text{MgSO}_4$  (0.005 M), 0.15 ml. of KCl (0.05 M), 0.30 ml. of ATP (0.0005 M), 0.30 ml. of phosphate buffer (0.01 M), 0.30 ml. of cytochrome *c* ( $1 \times 10^{-5}$  M), 0.15 ml. of sodium octanoate (0.001 M), 0.15 ml. of malate (0.0005 M), and water to make 3.0 ml. When KCl was introduced with the enzyme (supernatant), the KCl concentration added to the flask was compensated to give a final concentration of 0.05 M. Time, 50 minutes in Experiment I, 65 minutes in Experiment II.

Experiment No.	Enzyme source	Oxygen uptake	Acetoacetate formed
		micromoles	micromoles
I	Water suspension, complete system	10.8	
	Same, octanoate omitted	3.2	
	KCl-pptd. material, complete system	10.8	
	Supernatant, complete system	0.0	
	KCl-ppt + supernatant, complete system	10.3	
II	KCl-pptd. enzyme, washed once with 0.1 M KCl, complete system	8.3	3.5
	Same, octanoate omitted	2.6	0.0
	Malate omitted	1.3	0.9
	Cytochrome <i>c</i> omitted	0.8	0.3
	KCl omitted	1.1	0.1
	ATP "	0.0	0.0
	$\text{Mg}^{++}$ "	2.9	0.4

$\text{NaCl}$ ,  $\text{LiCl}$ , and sucrose also cause the flocculation of the enzyme complex from the *water* suspension.

## DISCUSSION

In earlier papers (16, 1) the senior author elaborated a working hypothesis for the observation of Lelou and Muñoz (20, 21) that the presence of adenylic acid and fumarate was required to demonstrate the oxidation of butyrate by a washed preparation of guinea pig liver. It was proposed that these two factors were necessary to cause the enzymatic generation of adenosine triphosphate from adenylic acid coupled to the oxidation of fuma-

rate and that ATP was actually the immediately necessary cofactor for fatty acid oxidation. As it happened, experiments based on this hypothesis proved highly successful in subsequent work (1), although the data presented in this paper make that hypothesis somewhat less tenable. Both adenine nucleotide and malate or oxalacetate appear to be required for enzymatic fatty acid oxidation, ATP is not capable of substituting for the two required compounds. This requirement of the system became obvious only after some manipulations of the enzyme, which resemble those employed by Leloir and Muñoz. The latter investigators apparently did not recognize fully the requirement for salts or non-electrolytes demonstrated in this paper, this factor may possibly account for the erratic activity of their enzyme preparations.

The finding that an intermediate of the Krebs cycle is required in addition to adenine nucleotide makes possible a closer correlation of the properties of the enzyme from guinea pig liver studied by Leloir and Muñoz, the fatty acid oxidase of heart muscle (22), and the oxidase system of rat liver studied here, all of which require both adenine nucleotide and malate or oxalacetate.

The mechanism of action of the required intermediate of the Krebs cycle, which our experiments tentatively indicate to be either malate or oxalacetate, is obscure and considerable work on this phase of the oxidation has not yielded fully definitive results. However, the data presented clearly indicate two separate functions for malate (or oxalacetate) in fatty acid oxidation, one, to help initiate the oxidation to the stage of acetoacetate (or the hypothetical 2-carbon intermediate), the second, to engage in condensation reactions with the 2-carbon intermediate to form tricarboxylic acid, thereby causing fatty acid oxidation to occur through the Krebs cycle via the 2-carbon unit stage (2).

The requirement of salts or certain non-electrolytes in approximately isotonic concentration for the preparation and activity of the fatty acid oxidase is an unusual finding in the light of known enzyme and protein properties. It is tempting to assume that these particles, catalyzing the reactions of the Krebs cycle and fatty acid oxidation with accompanying aerobic phosphorylations in a highly integrated manner, preexist as such in the intact liver cell. However, examination of isolated nuclei, mitochondria, and microsomes prepared by the method of Schneider (23) for fatty acid oxidase activity has given no positive results, all fractions being completely inactive in the test system described in this paper. This approach is complicated by the strong possibility that the enzyme system becomes inactivated during the course of the fractionation procedure.

#### SUMMARY

Particulate material separated from rat liver homogenized in isotonic salt solutions, washed with saline, and then suspended in *water* showed no

activity in the oxidation of fatty acids when supplemented with ATP,  $Mg^{++}$ , and phosphate buffer. When the material was suspended in *saline* instead, the preparations were highly active with the supplements named. However, the fatty acid oxidase activity of the *water* suspension of the particles could be completely restored in the presence of ATP,  $Mg^{++}$ , phosphate buffer, cytochrome *c*, neutral salts, or certain non-electrolytes such as sucrose, and catalytic amounts of malate or oxalacetate. The function of the inorganic phosphate was found to lie in its participation in coupled oxidative phosphorylation by the use of  $P^{32}$  as a tracer. The action of malate or oxalacetate has two distinct phases: one, to initiate the oxidation of octanoate to the stage of the 2-carbon intermediate, and the second, to cause the latter to enter into the Krebs cycle by forming tricarboxylic acid. The function of the neutral salts in the fatty acid oxidase system appears to lie in the production of an enzymatically active "flocculated" form of the enzyme from an inactive "dispersed" form.

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*Addendum*—Since this manuscript was prepared for publication, we have published a preliminary report (24) in which data were presented to show that the fatty acid oxidase activity is present exclusively in the mitochondria fraction of rat liver prepared by the method of Hogeboom, Schneider, and Pallade (25).

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## RAPID DETERMINATION OF *n*-OCTANOIC ACID\*

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The lack of rapid, specific methods for routine determination of the saturated fatty acids has been an impediment in the study of enzymatic reactions of fatty acids. The classical steam distillation and titration technique applied to the higher steam-volatile fatty acids, such as *n*-octanoic acid, in amounts likely to be used in tissue slice or enzyme experiments has been found to be completely inadequate on the basis of the labor and time involved in the distillations, the lack of specificity within the homologous series of steam-volatile fatty acids, and the uncertain accuracy. For instance, Leloir and Muñoz have used the distillation technique in the analysis of octanoic acid in tissue slice media and report consistently incomplete recoveries, with negative errors as large as 27 per cent, in the distillation and titration of 5 to 20 micromole quantities (1).

In this paper is described a method for rapid determination of *n*-octanoate which we have used extensively in this laboratory in the study of the enzymatic oxidation of fatty acids. The method responds only to normal saturated fatty acids having from 7 to 10 carbon atoms, as far as is known, and is analytically useful for *n*-octanoic and *n*-nonanoic acids. Higher and lower fatty acids give no response with this method, nor do any of a large series of compounds likely to be present in biological systems. The method to be described has an effective range of 2 to 7 micromoles of octanoate. In addition, details of a micro modification are given which allow determinations in the range of 0.4 to 1.4 micromole.

**Principle of Method**—A copper-lime filtrate containing the fatty acid is acidified and extracted with petroleum ether in a glass-stoppered centrifuge tube. The aqueous phase is removed with a capillary pipette. The petroleum ether extract is freed of interfering substances forming insoluble silver salts (chlorides, etc.) by washing with water. The fatty acid is then extracted into 0.1 *N* NaOH. A sample of the NaOH layer is brought to pH 5.6 with acetic acid and silver nitrate is added, forming a turbidity consisting of insoluble silver octanoate which is stabilized with gum ghatti. The optical density of the turbidity is proportional to octanoate concentration within limits and is measured in a photoelectric colorimeter.

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The extractions and washing are performed in a single vessel, a glass-stoppered, conical tipped centrifuge tube. Since the time factor is not critical in any phase of the determination, the number of samples which may be run simultaneously is limited by the capacity of the shaking apparatus, which is convenient but not essential for the analysis.

The specificity of the method for the saturated normal fatty acids having from 7 to 10 carbon atoms is due to two factors: fatty acids having less than 7 carbon atoms are less readily extracted from water solution by petroleum ether and have relatively soluble silver salts, and fatty acids having more than 10 carbon atoms form insoluble calcium or copper salts and are completely removed in the copper-lime deproteinization treatment.

#### *Reagents—*

1.  $\text{CuSO}_4$  solution    20.0 gm of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  per 100 ml of solution
2. Calcium hydroxide suspension    10 per cent suspension in water
3. Metaphosphoric acid solution    15.0 gm made to 100 ml
4. Skellysolve B (petroleum ether, stated by the manufacturer to be essentially *n*-hexane, boiling range 60–71°)
5. Sodium hydroxide solution adjusted to 0.100 *N*. This solution must be free of chloride ion.
6. Acetic acid-gum ghatti solution    13.0 ml of glacial acetic acid are diluted with approximately 150 ml of  $\text{H}_2\text{O}$ . To this solution are added 15 ml of 1 per cent gum ghatti solution. The mixture is made to 500 ml with  $\text{H}_2\text{O}$  and filtered. The gum ghatti solution is prepared by suspending 1.0 gm of gum ghatti (Sargent) in a gauze bag in 100 ml of  $\text{H}_2\text{O}$  overnight at room temperature, followed by filtration. The combined reagent is stable for as long as 6 weeks at room temperature. This solution must be chloride-free.
7. 1.5 *M* silver nitrate
8. Stock standard octanoate solution    1.58 ml of *n*-octanoic acid (Eastman, redistilled) are dissolved in 50 ml of warm 0.21 *N* NaOH with shaking and made to 100 ml with  $\text{H}_2\text{O}$ . This solution, which is 0.10 *M* (100 micromoles per ml) is kept in the refrigerator. Before use it is allowed to warm to 30° and shaken thoroughly.

#### *Apparatus—*

1. 40 ml glass-stoppered, conical tip centrifuge tubes. Maizel-Gerson reaction vessels (without stop-cock, supplied by the Wilkens-Anderson Company, 111 North Canal Street, Chicago) have been used in this laboratory.

2. Shaking machine. We have used the International No. 2 machine, adapted to hold sixteen tubes. It is recommended that the shaking times necessary for complete extraction of fatty acid into and out of Skellysolve B be determined for the particular machine and shaking rate used.

3 Capillary pipettes, drawn from glass tubing These are connected with rubber tubing to a ground glass stop-cock, which in turn is connected with flexible rubber tubing to a large suction flask and a water aspirator

4 40 ml transfer pipettes

5 Tubes to fit the photoelectric colorimeter The method as described was designed to yield 5.2 ml of suspension to be read in the Evelyn photoelectric colorimeter, with the 6 ml opening with standard Evelyn tubes

### Procedure

*Extraction of Octanoate into Skellysolve B*—An aliquot of protein-free filtrate (the deproteinization procedure is considered in a separate section) containing between 2 and 7 micromoles of octanoate (0.20 to 0.70 ml of 0.01 N acid) is placed in a 40 ml glass-stoppered, conical tip centrifuge tube and 1.0 ml of 15 per cent metaphosphoric acid is added The volume is made to 10.0 ml with  $H_2O$  To the mixture are added 10.0 ml of Skellysolve B The tubes are stoppered and shaken for 5 minutes The aqueous phase (lower layer) is then removed from each tube by means of the drawn out capillary pipettes attached via rubber tubing and a glass stop-cock to a water aspirator Removal of the aqueous layer can be made essentially complete, since the conical tip of the tube and the control provided by the glass stop-cock allow removal of all but a very small drop of the aqueous phase

*Washing of Skellysolve Extract*—To each tube are added 10.0 ml of  $H_2O$  (chloride-free), the water being allowed to rinse the surface of the ground glass joint The tubes are restoppered (the stoppers meanwhile having been rinsed in a stream of chloride-free water) and again shaken for 5 minutes The aqueous layer is removed as completely as possible, again with capillary pipettes and a suction line

*Extraction of Octanoate into Aqueous NaOH*—To each tube are added 5.0 ml of 0.100 N NaOH The tubes are stoppered and again shaken for 5 minutes

*Development of Turbidity*—A 4.0 ml sample of the NaOH layer is removed with a 4.0 ml transfer pipette The upper end of the pipette is closed with the pipette finger as the pipette is passed down through the upper layer of Skellysolve to prevent removing any solvent with the aqueous layer The pipette is wiped with gauze to remove adhering Skellysolve and the contents delivered into a colorimeter tube containing 1.0 ml of the acetic acid-gum ghatti solution The contents are mixed by shaking To each tube is added 0.20 ml of 1.5 M  $AgNO_3$ , causing the formation of a turbidity due to silver octanoate The contents are immediately mixed by shaking The turbidities are read in a photoelectric colorimeter with a 660  $m\mu$  filter The tubes are shaken just before the reading is taken

Ordinarily we have made the readings 15 minutes after addition of the silver nitrate, but the turbidities remain constant for an hour

*Colorimeter Blank*—The blank tube contains 4.0 ml of 0.1 *N* NaOH, 1.0 ml of the acetic acid-gum ghatti solution, and 0.20 ml of 1.5 *M* AgNO<sub>3</sub>. This tube is used to set the galvanometer (in the case of the Evelyn instrument) at 100

*Standard Curve*—A standard curve relating micromoles of octanoate to colorimeter readings is prepared for each new batch of reagents. The standard curve obtained can be consistently duplicated with the same batch of reagents, therefore in practice only a single standard is run with each set of determinations. The stock standard solution (100 micromoles per ml) is diluted 1/100 to prepare a working standard containing 1.0 micromole of

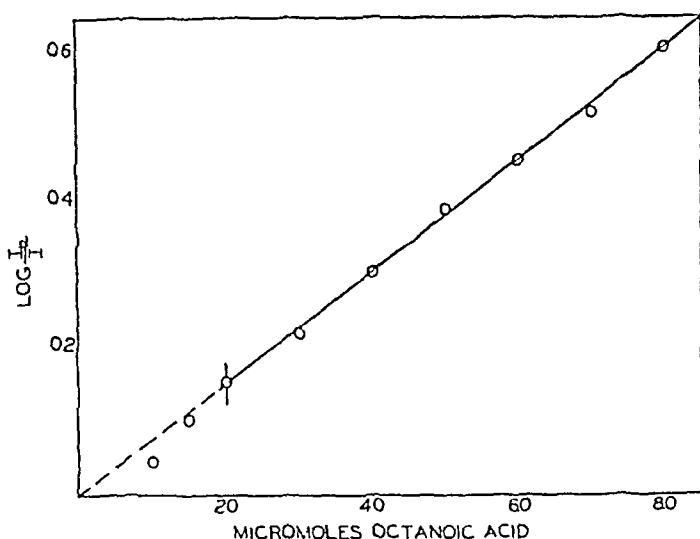


FIG. 1. Standard curve for determination of *n*-octanoic acid.

fatty acid per ml. Aliquots corresponding to between 1.0 and 8.0 micromoles are carried through the entire procedure, including the deproteinization steps. In Fig. 1 is shown a typical standard curve prepared for octanoic acid. Only the linear portion of the curve is used, since below 2.0 and above 7 to 8 micromoles of fatty acid the turbidity is no longer linear.

*Average Error of Single Determinations*—Several sets of experiments to test the reliability of single determinations and actual use of the method applied to studies of the fatty acid oxidase system (see the preceding paper) indicate that the average error of a single determination does not exceed 3.5 per cent. This figure compares very favorably with the values obtained by Lelon and Muñoz (1) for distillation and titration of octanoic acid in amounts ranging from 5 to 20 micromoles. They reported consistently incomplete recoveries, with errors ranging from -16 to -27 per cent.

*Deproteinization*—A study was made of various deproteinization procedures to determine the best method from the standpoint of completeness of recovery and elimination of interfering materials which cause formation of emulsions. The modified copper-lime treatment described below is the only one of many procedures tested which proved to be satisfactory. Trichloroacetic acid, tungstic acid, metaphosphoric acid, and heat coagulation were found to cause *n*-octanoic acid to become adsorbed on the coagulated protein to such an extent that in many cases no octanoic acid whatsoever could be recovered in the filtrate. This is not particularly surprising, since the fatty acids, having 7 or more carbon atoms, are very sparingly soluble in acid solution and are highly surface-active. However, the alkaline conditions prevailing in the copper-lime treatment favor complete recovery of heptanoic, octanoic, and nonanoic acids from protein-containing mixtures. Even with this method of deproteinization there are definite limitations to the conditions under which fatty acid may be recovered completely from protein-containing mixtures. For this reason conditions must first be established for complete recovery of fatty acid from the material to be analyzed.

The method has been used for the determination of fatty acid added as substrate to a buffered medium in which tissue slices were shaken in Warburg vessels. In such experiments no difficulty was experienced in recovering added octanoate. After completion of the experiment the slices were removed from the Warburg vessels and an aliquot of the medium removed for treatment as described below.

Fatty acid determinations in tissue suspensions or homogenates, however, are subject to definite limitations because the ratio of fatty acid concentration to tissue concentration is critical in obtaining complete recovery of fatty acid by the deproteinization procedure described below or by variations thereof. In Table I are shown recoveries of a standard amount of octanoate from a standard volume of mixture containing variable amounts of rat liver homogenized in water. It can be seen that as the tissue concentration is increased a point is reached at which the recovery of a known amount of octanoate falls off considerably.

In general, octanoate may be recovered quantitatively from suspensions containing as much as 10 per cent tissue, provided the suspension is diluted with at least an equal volume of water before application of the copper-lime treatment. The concentrations of copper sulfate and calcium hydroxide suspension used in the method as described below are optimal, increasing these concentrations or altering their ratios does not lead to increased recovery at very high tissue concentrations.

In Table II are shown recoveries obtained in a case in which conditions were satisfactory for complete recovery of a wide range of octanoate con-

centiations The experiments described in Tables I and II may be used as a guide in establishing the proper deproteinization method when analysis of fatty acid in given enzyme preparations or bacterial suspensions is desired

TABLE I

*Effect of Tissue Concentration on Recovery of Standard Amounts of n-Octanoic Acid from Rat Liver Homogenate*

Each tube contained 1.00 ml of octanoate (12.0 micromoles), water, and rat liver homogenate to make a total volume of 6.0 ml. Immediately after mixing, 6.5 ml of H<sub>2</sub>O were added, followed by 1.0 ml of CuSO<sub>4</sub> solution and 1.50 ml of Ca(OH)<sub>2</sub> suspension. Octanoic acid recovery was determined in aliquots of the filtrate. The liver homogenate contained 250 mg of fresh tissue per ml.

Volume of homogenate added	Octanoic acid recovered	Volume of homogenate added	Octanoic acid recovered
<i>ml</i>	<i>micromoles</i>	<i>ml</i>	<i>micromoles</i>
0.0	12.1	2.5	10.9
0.5	12.0	3.0	10.2
1.0	12.0	4.0	4.1
1.5	11.8	5.0	2.0
2.0	12.2		

TABLE II

*Recovery of Octanoate from Rat Liver Homogenate under Favorable Conditions*

Each tube contained 4.0 ml of octanoate (0 to 24.0 micromoles, as indicated) and 2.0 ml of rat liver homogenate (250 mg of tissue per ml). Immediately after mixing, 6.5 ml of H<sub>2</sub>O were added, followed by 1.0 ml of CuSO<sub>4</sub> and 1.50 ml of Ca(OH)<sub>2</sub> suspension. Octanoic acid was determined in duplicate on 5.0 ml aliquots of filtrate.

Octanoic acid added	Octanoic acid recovered	Octanoic acid added	Octanoic acid recovered
<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>	<i>micromoles</i>
24.0	24.4	10.0	10.2
	24.5		9.8
18.0	17.9	9.0	9.0
	17.6		8.8
15.0	15.3	6.0	5.8
	14.8		5.8
12.0	12.0	0.0	0.0
	11.8		0.0

The deproteinization treatment found best for analysis of fatty acid in tissue slice media, tissue homogenates, and washed suspensions of ground tissues (2) follows. The sample, containing between 6.0 and 21 micromoles of octanoate, is diluted with water to 12.5 ml. 1 ml of the CuSO<sub>4</sub> solution

is added and mixed, and this is followed by the addition of 15 ml of  $\text{Ca}(\text{OH})_2$  suspension. The contents are thoroughly mixed and allowed to stand 15 minutes. The tubes are centrifuged and the supernatants filtered through paper. The volume of filtrate is enough for analysis of two aliquots of 5.0 ml, containing from 2.0 to 7.0 micromoles of fatty acid, which are carried through the procedure as already outlined.

Metaphosphoric acid is used as the acidifying reagent in the extraction procedure because it serves to prevent formation of emulsions due to traces of protein which escape removal in the copper-lime treatment.

TABLE III

*Specificity in Homologous Series of Saturated Normal Acids*

The indicated amounts of the following fatty acids were carried through the entire analytical procedure including the copper-lime treatment, and the turbidities resulting were expressed in terms of the micromoles of octanoate giving equal turbidity readings.

Fatty acid	No. of C atoms in chain	Amount in sample	Octanoic acid equivalent
		<i>micromoles</i>	<i>micromoles</i>
	2	1000	No turbidity
	4	300	" "
	5	500	0.9
	6	50	0.6
	7	15	3.4
		10	No turbidity
	8	5	5.0
	9	5	3.7
	10	5	2.1
	12	200	No turbidity
	14	200	" "
	16	200	" "

*Specificity*—The following observations indicate the specificity of the method. All normal saturated fatty acids up to and including *n*-nonanoic acid may be completely recovered in the copper-lime treatment outlined above. *n*-Decanoic acid cannot be completely recovered, when 10 micromoles of *n*-decanoate were subjected to the copper-lime procedure outlined, only 4.5 micromoles could be recovered. Fatty acids having 12 or more carbon atoms are *completely* removed by the copper-lime procedure, even when relatively large amounts are present owing to the insolubility of their copper or calcium salts. Of the fatty acids having fewer than 10 carbon atoms only octanoic and nonanoic acids can be determined by the method outlined, since only these acids show a linear relationship between optical density and concentration. Although 15 micromoles of heptanoic acid give

a response equal to that given by 3.4 micromoles of octanoate, there is no linearity of response. Fatty acids having less than 7 carbon atoms must be present in relatively enormous concentration to yield even a slight turbidity. The data presented in Table III indicate the magnitude of turbidity responses given by different fatty acids.

In order to test the specificity of the method with respect to compounds other than saturated fatty acids, mixtures containing 1.0 ml of 15 per cent metaphosphoric acid, 5.0 ml of octanoate (5 micromoles) or 5.0 ml of  $H_2O$ , and 4.0 ml of a solution containing 150 micromoles of the substance to be tested for interference were carried through the entire procedure (the deproteinization step was omitted). The following compounds gave absolutely no turbidity under these conditions nor did they interfere with the recovery of octanoate: sodium chloride, sodium sulfate, magnesium sulfate, monosodium phosphate, sodium borate, sodium fluoride, potassium iodoacetate, ammonium sulfate, sodium arsenate, trichloroacetic acid, tungstic acid, glucose, fructose, xylose, glycogen, glycine, cysteine, acetylcholine, *p*-aminobenzoic acid, aniline acetate,  $\alpha$ -aminooctanoic acid, ascorbic acid, alloxan, sodium benzoate, glycolic acid, glycerol, creatine, ethyl alcohol, acetaldehyde, lactate, pyruvate, acetylpyruvate, citrate,  $\alpha$ -ketoglutarate, succinate, fumarate, *l*-malate, oxalacetate, *cis*-aconitate, acetoacetate, acetate, malonate,  $\beta$ -glycerophosphate, fructose diphosphate, sodium pyrophosphate, adenosine triphosphate,  $\beta$ -hydroxybutyrate,  $\beta$ -hydroxyoctanoate, crotonate, sorbate,  $\Delta^1$ - and  $\Delta^2$ -hexenoate,  $\alpha,\gamma$ -diketooctanoate, acetoin, *l*-glutamate, and glutamine.

The responses given by unsaturated acids of intermediate chain length, branched chain acids, or halogenated acids have not been more extensively examined. If such compounds are suspected to be present in the material to be analyzed, the specificity of the response must of course be examined further.

*Efficiency of Extractions*—The extractions of octanoate from the aqueous phase and from Skellysolve back into aqueous NaOH are essentially complete. This was determined by preparing a series of turbidities developed from octanoate in 0.1 *N* NaOH directly, without going through the extraction procedure, and comparing the responses given with those obtained when the same amounts of octanoate were carried through the whole procedure, allowance being made for the aliquot of NaOH extract used for turbidity development. The standard curves obtained in the two cases are identical. This indicates that the extractions are complete and that washing the extract with water does not cause any measurable loss of fatty acid.

*Effect of Variations in Volume of Aqueous Phase Extracted*—Although the volume of the aqueous phase extracted has been specified as 10.0 ml, it

may be varied between 5.0 and 20.0 ml with essentially complete recoveries. Use of a large aqueous phase may be necessary for analysis of filtrates in which the fatty acid concentration is very low.

*Efficiency of Washing Procedure*—The conditions of analysis suggest that a possible point of difficulty might lie in the washing of the Skellysolve extract to free it of chlorides or other substances which form insoluble silver salts. When such substances are present in the concentrations likely to be found in biological fluids or in enzyme reaction media, absolutely no interference has been encountered. When 9.0 ml of saturated NaCl solution acidified with 1 ml of metaphosphoric acid were carried through the analysis, only a faint turbidity (due to AgCl), corresponding to less than 0.4 micromole of octanoate, was observed. The failure of chlorides in ordinary concentrations to produce turbidities indicates that the washing of the Skellysolve extract containing the fatty acid is extremely efficient in removing traces of the original aqueous phase from the centrifuge tubes used in the analyses. We have never experienced any difficulties due to contamination of reagents or glassware with chlorides or other substances capable of reacting with silver ions.

*Conditions Affecting Turbidity Response*—The pH of the acetate-buffered solution in which the turbidity is developed is 5.6. It has been found that the medium must be well buffered to obtain reproducible turbidities. Raising the pH substantially causes silver acetate to be coprecipitated and the optical densities of the turbidities given by standard amounts of octanoate are no longer linear. Lowering the pH causes, as would be expected, a great decrease in the sensitivity, since the silver octanoate becomes more soluble. The concentration of acetate chosen is high enough to give maximal stability of response without causing any interference by precipitation of silver acetate.

The omission of gum ghatti from the turbidity mixture greatly reduces the stability of the turbidity and also reduces the useful range in which optical density is linear with octanoate concentration, since at the extremes the silver octanoate flocculates out immediately or after short standing. Gum arabic, agar, soluble starch, and gelatin have also been tested but gum ghatti is the most effective agent for stabilizing the turbidities. When the concentration of gum ghatti is greatly increased, the range of response is decreased, since the lower concentrations of octanoate then fail to produce a turbidity with silver ions.

The concentration of silver ions is also critical in the optical density produced, although 10 per cent errors in the addition of the silver nitrate solution cause no significant change in optical density. Substantial decreases in concentration of silver ion cause concomitant decreases in sensitivity, since the solubility product of silver octanoate is not exceeded with the



lower range of octanoate concentrations. Increasing the concentration of silver ion brings about an increasing tendency for silver acetate to precipitate after short standing.

Attempts to combine the silver nitrate and the acetic acid-gum ghatti reagents for greater convenience in analysis met with failure, since addition of such a combined reagent to the 0.1 *N* NaOH solution of octanoate causes precipitation of silver oxide which does not immediately redissolve on mixing, owing probably to the presence of the gum ghatti. The turbidities developed under these conditions have been found to be very erratic.

The turbidities of silver octanoate are not photosensitive over a period of several hours and no special precautions have been found necessary to control this factor.

*Micro Modification*—Although the method as outlined above has been found to be quite suitable in routine analysis of large numbers of samples, in certain cases the small size of the samples available did not permit use of the method in the useful range. For contingencies of this type we have devised a modification in which the working procedure is identical but in which volumes are reduced in scale by a factor of 5. Samples containing 0.4 to 1.4 micromole of octanoate are acidified with 0.2 ml. of  $\text{HPO}_3$  and made to 2.0 ml. They are extracted with 2.0 ml. of petroleum ether in a 10 ml. conical tipped tube, the extracts washed with 2.0 ml. of  $\text{H}_2\text{O}$ , and the octanoate brought into 1.0 ml. of 0.1 *N* NaOH. An 0.8 ml. sample of the NaOH layer is added to 0.2 ml. of the acetic acid-gum ghatti and the resulting mixture is treated with 0.04 ml. of 1.5 *M*  $\text{AgNO}_3$ . The turbidities are read at 660  $m\mu$  in a Beckman quartz spectrophotometer equipped with a diaphragm adapter to produce a "pinhole" light source (3). The position of the beam was adjusted so that it passed through a standard 3.0 ml. Beckman cell mounted on an adjustable block in the cell holder without striking the bottom or walls of the cell or the meniscus when the cells contained 1.0 ml. samples. The general principles outlined by Lowry and Bessey (3) for microspectrophotometric measurements were observed. The errors were not significantly greater than in the macro method.

#### SUMMARY

A simple method for the rapid determination of octanoic acid in small amounts has been described. The method involves deproteinization with mixtures of copper sulfate and calcium hydroxide, acidification of the filtrate, and extraction of the fatty acid into petroleum ether. The extract is washed with  $\text{H}_2\text{O}$  and the fatty acid then extracted into 0.1 *N* NaOH. An aliquot of the aqueous phase is brought to pH 5.6 by addition of acetic acid. The addition of silver ions produces a turbidity of silver octanoate, stabilized with gum ghatti, the optical density of which is proportional to oc-

tanoic acid concentration. The method responds only to *n*-octanoic acid and immediately adjacent homologues. A large variety of compounds likely to be present in enzyme reaction media were found not to interfere with the determination.

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## PREPARATION OF RADIOACTIVE CARBON-LABELED SUGARS BY PHOTOSYNTHESIS\*

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Carbohydrates, glucose, fructose, sucrose, and starch, labeled in all carbon positions with  $C^{14}$ , are useful for the study of intermediate metabolism in living organisms and for many other purposes. Two papers dealing with the synthesis of labeled carbohydrates have already appeared (1, 2), but no detailed methods of preparing the above carbohydrates are available. It is the purpose of this paper to describe such methods.

Radioactive starch, glucose, fructose, and sucrose can be isolated from green leaves in good yields after they have been exposed to an atmosphere of radioactive carbon dioxide in the presence of light. At the end of the photosynthetic period, the leaf or leaves are extracted with dilute alcohol. The alcohol-insoluble fraction contains the starch which can be isolated. Hydrolysis of the starch with acid produces glucose which can be obtained in crystalline form. The alcohol extract contains glucose, fructose, and sucrose. In order to increase the yield of the monosaccharides, the sucrose is hydrolyzed with acid, thus producing additional glucose and fructose. The separation of fructose from glucose is accomplished by precipitating the former with calcium hydroxide. The calcium complex is then decomposed and the fructose liberated. Sucrose is separated from the alcoholic extract. The two monosaccharides are first fermented with *Torula monosa* and then the residual sucrose is concentrated and crystallized.

**Apparatus**—The photosynthetic chamber is made of a Pyrex glass tube (Fig. 1) 21 cm. in length having an inside diameter of 5.5 cm. The lower end consists of a drawn down, recurved entry tube fitted with a stop-cock,  $a$ , and a 10/30 tapered joint. The upper end bears a 60/50 joint,  $A_1$ , to which a cover is fitted, carrying an exit tube which is also fitted with a

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stop-cock, *b*, and a 10/30 joint. The volume between stop-cocks *a* and *b* is 500 ml.

When conducting an experiment, about 500 mg of barium carbonate containing approximately 0.25 millicurie of  $C^{14}$  are introduced into a 25 ml Erlenmeyer flask, *B*, from which the neck has been removed. A cork disk, *c*, slightly smaller than the inside diameter of chamber *A*, is fitted to the top of the flask so that its position is fixed when placed in the chamber. The barium carbonate is then mixed with about 5 ml of water and a few drops of paraffin oil are added to prevent excessive foaming. 1 ml of 80 per cent lactic acid is placed in a small test-tube, *f*, cut off so that it rests at about a 45° angle when placed in flask *B*. This flask, containing the barium carbonate slurry and the test-tube of lactic acid, is then placed in an upright position in the photosynthetic chamber, *A*, which was previously rinsed

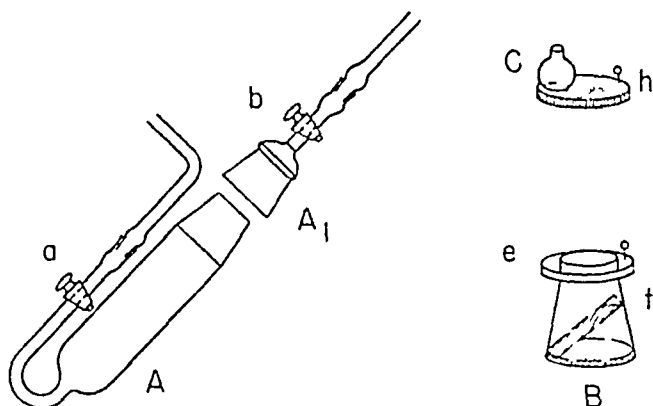


FIG. 1. Photosynthetic chamber, Erlenmeyer flask, and Florence flask.

with water to assure a humid atmosphere. Enough water should also be left in the receiving portion of the entry tube to indicate when the internal and external pressures are equalized.

A 10 ml Florence flask, *C*, from which the neck has been removed is attached to a plywood disk, *h*, bored through with numerous holes. The flask is filled with water and the petiole of the leaf, which had previously been kept in the dark for 24 hours, is inserted so that it reaches the bottom of the flask. The leaf and its container are placed in a vacuum desiccator and evacuated to about 20 cm. After the initial flow of gas bubbles from the petiole of the leaf ceases, the pressure is equalized and the water displaced in flask *C* is replaced. The leaf and container are then placed in the photosynthetic chamber, *A*, on top of the cork ring, *c*. A piece of moistened filter paper is placed over the plywood disk to prevent spattering of acid into the upper part of the vessel. The greased, upper end, ground taper, *A1*, is fitted on, and with the entry tube, *a*, closed, the chamber is partially

evacuated through the exit tube which is then closed with stop-cock *b*. By tilting the chamber about  $30^\circ$  the acid is dumped into the barium carbonate slurry. This should be done carefully to avoid excessive foaming. When the reaction has subsided and all the barium carbonate has reacted, liberating the carbon dioxide, atmospheric pressure is restored by opening stop-cock *a*. The chamber is then immersed in a cylindrical Pyrex water bath, 10 inches high and 18 inches in diameter, held in position by a condenser clamp on a heavy ring-stand in the bath as shown in Fig. 2.

Illumination is effected by two 100 watt bulbs in desk lamps,  $g_1$  and  $g_2$ , placed opposite each other on the outside of the bath. A small fan (not shown in Fig. 2) is placed above the water bath so that a current of air passes over the surface of the bath. This maintains the bath temperature  $3-4^\circ$  above the prevailing room temperature. Illumination is continued for

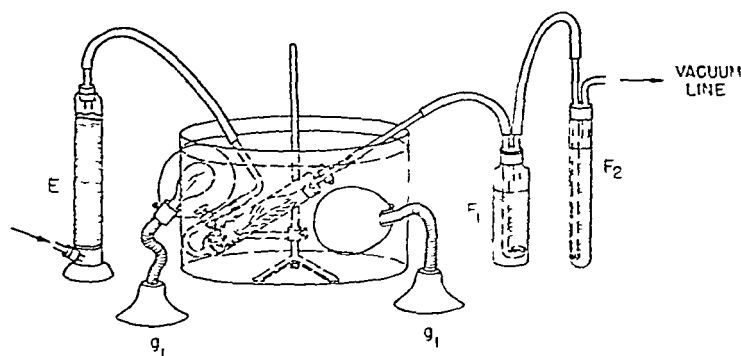


FIG. 2 Complete apparatus for photosynthesis

18 to 24 hours. A relatively long period of illumination is used in order to insure complete utilization of the available carbon dioxide and to produce more uniformly labeled compounds.

During the last hour of illumination a soda lime tower, *E*, is attached to the entry tube of the photosynthetic chamber and two carbon dioxide traps, *F*<sub>1</sub> and *F*<sub>2</sub>, are attached in series to the exit tube. During this period about 5 liters of carbon dioxide-free air are pulled through the system by applying a vacuum at *F*<sub>2</sub>. Trap *F*<sub>1</sub> consists of a 500 ml jar fitted with a sintered glass aerator containing 335 ml of 0.1 *N* sodium hydroxide, trap *F*<sub>2</sub> consists of a 100 ml test-tube also equipped with an aerator and 65 ml of 0.1 *N* sodium hydroxide.

Usually no appreciable amount of carbon dioxide is found in traps *F*<sub>1</sub> and *F*<sub>2</sub> at the end of the illumination period.

*Plant Materials*—Turkish tobacco leaves proved to be good starch producers. A high rate of synthesis can be achieved after the leaves are

placed in the dark for approximately 24 hours to use up the reserve carbohydrate and are then subjected to photosynthesis in an atmosphere initially containing 10 to 15 per cent carbon dioxide. It has been observed that under the experimental conditions described about 20 per cent of the dry weight of the alcohol-extracted leaf residue consists of starch.

The plants are grown in Hoagland's solution or sand culture. The leaves, weighing from 3.0 to 3.5 gm. and being from 15 to 18 cm. in length, are taken from the middle portion of the stem of plants about 2.5 feet high. Leaves of plants grown in culture solution are harvested in the morning and placed in water in the dark until the following morning. Plants grown in pots of sand are placed with the container in the dark for a similar period of time and the leaves harvested just prior to the experiment. On a fresh weight basis the starved leaves usually contain about 0.25 per cent reducing sugars (glucose and fructose) and 0.15 per cent sucrose. The residue does not give a blue color with iodine, indicating the absence of starch. After 24 hours of illumination in the chamber in an atmosphere of carbon dioxide derived from 0.5 gm. of barium carbonate, about 0.85 per cent reducing sugars, 0.65 per cent sucrose, and 2.5 per cent starch are found.

*Labeled Starch*—Several lots of radioactive starch were prepared by the following method. A Turkish tobacco leaf was harvested in the morning and allowed to remain in the dark for 24 hours to exhaust the starch. The leaf was then placed in the photosynthetic chamber in an atmosphere of radioactive carbon dioxide, as previously described, and illuminated for 24 hours. At the conclusion of the photosynthetic period and after the chamber had been swept out with carbon dioxide-free air, the leaf was killed by immersion in boiling 80 per cent alcohol. It was then cut into small pieces and placed in a Soxhlet extraction thimble. The alcohol used in killing the leaf was transferred to a boiling flask of a Soxhlet extractor. The extractor was assembled and the extraction continued for 6 to 8 hours, after which the alcoholic extract containing the soluble sugars (glucose, fructose, and sucrose) was set aside.

After the 80 per cent alcohol extraction, the residue remaining in the Soxhlet thimble was dried at 50° in a vacuum oven for a period of 18 to 24 hours (0.31 to 0.42 gm. of dry material was obtained), 25 per cent of its weight of magnesium carbonate was added, and the mixture finely ground in a mortar under a hood. The ground material was extracted by the method of Pucher and Vickery (3) as follows. It was transferred to a heavy duty 50 ml. centrifuge tube to which an amount of 100 to 150 mesh ground glass, equivalent to 7.5 times the weight of the plant material, was added. This was followed by the addition of 5 ml. of water and a heavy stirring rod was placed in the tube. The tube with the contents was immersed in a steam

bath and vigorously stirred<sup>1</sup> for 15 minutes to gelatinize the starch 7 ml of 46 per cent calcium chloride, previously heated on the same water bath, were added and stirring was continued for 10 minutes The tube was centrifuged, the supernatant liquid decanted into a 100 ml centrifuge tube, and the residue ground again for about 5 minutes The tube was returned to the steam bath, 3 ml of boiling water and 5 ml of 46 per cent calcium chloride at 100° were added, and stirring continued for 10 minutes The tube was centrifuged and the supernatant liquid decanted into the 100 ml tube containing the first extract After four such extractions, the last drop of the fourth supernatant liquid was acidified with a drop of 0.5 N acetic acid and tested with dilute iodine solution If no blue starch-iodine color was observed, the extraction of starch was assumed to be complete and the residue was washed twice with 15 ml of hot water If the starch-iodine test was positive, the extraction procedure was continued until a negative test was obtained Seven or eight extractions are sometimes necessary to free the residue of starch completely

The extract (60 to 120 ml) in the 100 ml centrifuge tube (or tubes) was treated with 1 ml of 2.3 N hydrochloric acid and 2 ml of 20 per cent sodium chloride per each 10 ml of extract The starch was then precipitated by the addition of 0.5 ml of iodine-iodide solution (30 gm of iodine and 50 gm of potassium iodide diluted to 250 ml) per 10 ml of extract The tube was loosely stoppered and allowed to stand for 10 minutes, after which it was placed in a steam bath for 15 minutes The tube and contents were cooled, centrifuged, the supernatant liquid decanted, and the starch-iodine complex well washed with 60 per cent alcohol The complex was then decomposed with 2 to 4 ml of 0.25 N alcoholic sodium hydroxide and washed three times with 60 per cent alcohol The crude starch was dissolved in 5 ml of water, filtered through a filter paper into a centrifuge tube, and reprecipitated with 1½ volumes of 95 per cent alcohol When precipitation was complete, the starch was centrifuged, washed with 60 per cent alcohol,<sup>2</sup> twice with 95 per cent alcohol, twice with absolute alcohol, and finally twice with ether After the ether had evaporated and the starch was ground to a powder, it was placed in a vacuum oven and dried at 50° for 24 hours

Several starch preparations made by this method with approximately

<sup>1</sup> The extraction of the starch from the plant material can also be effected by homogenization as described by Umbreit, Burris, and Stauffer (4)

<sup>2</sup> If the starch is to be used immediately for preparing radioactive glucose, the drying is unnecessary, it can be immediately hydrolyzed with sulfuric acid after washing with 60 per cent alcohol, as described in "Preparation of labeled glucose from starch"



230 microcuries of  $C^{14}$  in 0.5 gm of barium carbonate yielded from 20 to 25 per cent starch calculated on a dry basis of the alcohol-extracted plant material. The specific activity of the starch was from 0.48 to 0.72 microcurie per mg.

Radioactivity was measured by spreading a weighed amount of the starch on an aluminum plate with the aid of alcohol and, after drying at  $50^\circ$  *in vacuo* for 24 hours, assaying the sample with a bell jar type counter, as described by Kamen (5).

*Preparation of Labeled Glucose from Starch*—The radioactive starch was hydrolyzed to glucose by dissolving it in sufficient water to make a 0.2 per cent solution, at the same time adding an amount of inactive glucose carrier necessary to provide enough material to crystallize conveniently (0.3 gm was usually added). An equal volume of 2 N sulfuric acid was added to this solution and the mixture refluxed for 30 minutes on a hot-plate. After cooling, the theoretical amount of powdered barium carbonate needed to neutralize the acid was added and the precipitate centrifuged off and washed. The supernatant liquid and the washings were then passed through Duolite ion exchange columns, C-3 and A-3, having 25 ml bed volumes. Each column was washed with 100 ml of water. The demineralized solution, about 300 ml, was concentrated to a small volume *in vacuo* at  $50^\circ$ , transferred to a 25 ml beaker, and taken to a thick sirup in the vacuum oven at  $50^\circ$ . The sirup was warmed on the steam bath and approximately 4 volumes of hot absolute alcohol were stirred in. To the resultant viscous mass 50 mg of finely powdered crystalline glucose were stirred in and the beaker was allowed to cool in a desiccator. Crystallization of the glucose was complete within 24 hours. The crystals were transferred to a sintered glass funnel with cold absolute alcohol, sucked dry, washed with ether, and placed in the vacuum oven at  $50^\circ$ . After 24 hours the sugar was ground in a mortar, weighed, and assayed for radioactivity.

An 85 mg starch sample, having a specific activity of 0.57 microcurie per mg and a total activity of  $48.6 \pm 5.9$  microcuries, yielded, after addition of 350 mg of inactive glucose, 406 mg of glucose with a specific activity of 0.09 microcurie per mg and a total activity of  $36.7 \pm 0.63$  microcuries.

Another 71 mg starch sample with a specific activity of 0.48 microcurie per mg yielded, after addition of 350 mg of inactive glucose, 383 mg of glucose having a specific activity of 0.1 microcurie per mg.

*Preparation of Labeled Fructose*—The 80 per cent alcohol plant extract contains glucose, fructose, and sucrose. Most of the labeled glucose can be crystallized out by adding inactive glucose to the mixture after acid hydrolysis of the sucrose. The fructose, which does not readily crystallize and remains in solution, can be separated from glucose by forming the insoluble calcium-fructose complex, which is later decomposed.

The technique used in the preparation of fructose was as follows. The 80 per cent alcoholic extract was concentrated on the steam bath with occasional additions of water until all the alcohol was removed. The aqueous solution was cooled and twice extracted, with a separatory funnel, with one-fourth of its volume of ether to remove pigments and tarry matter. The ether was then washed with water and the washings added to the original aqueous phase. This latter solution was returned to the steam bath and heated to remove the dissolved ether. About 400 mg of inactive glucose carrier were added, and, after the solution was cooled, it was passed through Duolite ion exchange columns, C-3 and A-3, of 25 ml bed volume capacity, to remove the organic acids and amino acids. Approximately 100 ml of wash water was used for each column, and the resulting neutral solution concentrated under reduced pressure at 50° to a volume of 8 ml. 2 ml of 5 N sulfuric acid were added and the solution heated in a water bath at

TABLE I  
*Results of Crystallizations of Glucose Solution*

Crop of glucose	Weight	Specific activity	Total activity
	mg	microcurie per mg	microcuries
1st	426	0.089	38
2nd	79	0.113	9
3rd	221 (Includes 200 mg carrier)	0.037	8.1

80° for 10 minutes to invert the sucrose. After cooling and diluting to about 50 ml, the solution was passed through the anion exchange column, A-3, to remove the acid used for hydrolysis. The resulting neutral solution of glucose and invert sugar was concentrated under a vacuum at 50° to a volume of 10 ml, transferred to a small beaker, and concentrated to a thick sirup in the vacuum oven. The glucose was then crystallized as previously described. When the mother liquor was concentrated, a second crop of crystalline glucose was obtained. The specific activity of this glucose was about 25 per cent greater than that of the first crop, indicating radioactive contamination. 200 mg of inactive glucose were added to the mother liquor in order to reduce the activity of the residual glucose. Upon crystallization, 221 mg of glucose were recovered with a specific activity equivalent to one-third of that of the second crop. Considering the dilution with inactive glucose, approximately one-tenth of the previous activity would be expected. The higher activity indicates that some radioactive fructose was carried down with the inactive glucose (see Table I).

The radioactive glucose obtained from the mixture with fructose was contaminated with about 1 to 2 per cent radioactive fructose. It could be

feed almost completely from the radioactive contaminant by adding inactive fructose and recrystallizing

The calcium-fructose complex was prepared according to Bates and his associates (6). 1 gm of fructose carrier was added to the mother liquor after practically all of the radioactive glucose had been crystallized out by the addition of inactive glucose and the alcohol present removed by evaporation on the steam bath. In order to precipitate this quantity of fructose, an amount of calcium oxide equivalent to half of the weight of the fructose should be added. A 19 per cent lime suspension was made up from 0.76 gm of calcium hydroxide powder and 3.25 ml of water, making a total weight of about 4 gm. The calcium hydroxide used was shown to contain 66 per cent calcium oxide, as determined by titration with 0.1 N hydrochloric acid. The sugar solution containing the radioactive fructose was diluted with 11.7 ml of water so that the fructose would constitute six per cent by weight of the total reaction mixture.

The fructose solution and the calcium oxide slurry were placed in a refrigerator at 0°. When the fructose solution was cooled to 0°, one-fourth of the lime slurry was poured into a 50 ml centrifuge tube in an ice bath and then one-fourth of the sugar solution was slowly added with vigorous mechanical stirring. After 15 minutes fine crystalline needles of calcium-fructose could be observed under the microscope. The second quarter of the slurry was added with continuous stirring, followed by the slow addition of the second quarter of the sugar solution. The third and fourth portions were added at 20 minute intervals and after an additional 20 minutes stirring the mixture was placed in a refrigerator overnight. The next day the precipitate was centrifuged in a chilled centrifuge cup and washed twice with 2 ml portions of ice-cold saturated calcium hydroxide.

The calcium-fructose complex was then treated with an excess of 1 M oxalic acid, with phenolphthalein as an indicator, and stirred with a mechanical stirrer. The calcium oxalate was centrifuged off and washed twice with 25 ml portions of water. The supernatant liquid and washings were then passed over ion exchange columns, Duolite C-3 and A-3, concentrated in a vacuum below 50° to a small volume, and finally taken to a sirup in a vacuum oven.

This sirup, containing practically all fructose, was crystallized according to Fischer and Baer (7). It was taken up in a small amount of warm absolute alcohol, a few ml of dry benzene were added, and the mixture was taken to dryness under reduced pressure, while a stream of dry air blew over the surface of the liquid. After repeating this procedure four times, the residue was taken up in a minimum quantity of hot absolute methyl alcohol, cooled in a desiccator, seeded with 50 mg of finely powdered crystalline fructose, and the fructose precipitated by the slow dropwise addition

of absolute ether. The crystals were allowed to remain in a desiccator for 24 hours, transferred to a sintered glass crucible with a mixture of equal volumes of methanol and ether, filtered off, and washed with ether. A yield of 0.925 gm of fructose was recovered having a specific activity of 0.0325 microcurie per mg (Table II).

Initially there was a total of 132 microcuries in the sugar solution to which 2.125 gm of inactive glucose and fructose were added. The radioactive sugars synthesized in the plant probably amounted to about 100 mg. These preparations were made from the combined 80 per cent alcoholic extracts of two tobacco leaves.

The extent to which glucose and fructose were contaminated with one another was found from a control experiment performed as follows. A tobacco leaf, which was previously placed in the dark and then illuminated in

TABLE II  
*Recovery from Preparations of Tobacco Leaves*

Recovered sugar	Amount recovered	Total
	<i>mg</i>	<i>microcuries</i>
Glucose	726	55
Fructose	925	30
Calcium-fructose complex, supernatant solution, calculated from reducing value	61	16.4
Calcium-fructose complex, wash water, calculated from reducing value	29	6.5
Total	1741	107.9

the presence of inactive carbon dioxide for 24 hours, was extracted with alcohol, the alcoholic extract clarified, and the sucrose hydrolyzed. The solution containing glucose and fructose was divided into two halves. To one-half of the solution, labeled fructose was added and the glucose crystallized as previously described. The other half was treated with labeled glucose and the fructose was isolated from the mixture by the calcium precipitation method.

The results indicated that the glucose crystallized from the mixture with labeled fructose contained 1.3 per cent fructose. The fructose was found to be contaminated with glucose to the extent of 2.5 per cent. The glucose was freed from the radioactive fructose contaminant almost completely by dissolving the sugar in water, adding inactive fructose, and recrystallizing. The glucose contaminant in the fructose was reduced to a negligible amount by dissolving the sample and oxidizing with barium hypoiodite solution as described by Goebel (8), passing the solution through ion exchange columns,

and recovering the fructose by concentrating the solution and recrystallizing

*Preparation of Labeled Sucrose*—For the production of sucrose a leaf from *Canna indica* was used. Leaves from this plant do not form any detectable starch but do produce a considerable amount of sucrose. Photosynthesis was carried out at 17°, since it was observed that the sucrose-reducing sugar ratio was more favorable at that temperature than at 28°. The ratio of sucrose to reducing sugars was 1.0 at 17°, whereas it was 0.7 at 28°.

A canna leaf with a blade from 12 to 15 cm. in length was picked from a new shoot on the rhizome, the petiole being cut to 2 cm. Such a leaf contains enough anthocyanin pigments nearly to mask the green color of the chlorophyll. Under the conditions of the experiment, when 500 mg. of barium carbonate were used at 17° with a 24 hour illumination period, all of the carbon dioxide was utilized. On a wet weight basis, the final level of reducing sugars was 1.4 per cent and that of sucrose 1.8 per cent.

The wet weight of the leaf, after it had been placed in the dark for 24 hours and then infiltrated with water through the petiole, was 4.92 gm. The leaf was placed in the photosynthetic chamber containing 227 microcuries of C<sup>14</sup> in 2.5 mm. of carbon dioxide, obtained from 0.5 gm. of barium carbonate. After 24 hours of illumination at 15°, the leaf was killed with alcohol as previously described and then refluxed overnight with 80 per cent alcohol.

The extract was concentrated on the steam bath, water being added from time to time to evaporate the alcohol, and the aqueous solution was extracted with ether in a separatory funnel. 200 mg. of sucrose carrier were added, the excess ether was evaporated on the steam bath, and the resultant aqueous solution passed over ion exchange columns. The neutral solution was concentrated to about 7 ml. and the reducing sugars fermented out with *Torula monosa*.

*Torula monosa* ferments glucose, fructose, and mannose, but does not attack sucrose or other disaccharides. The organism is grown aerobically for 24 hours at 28° on agar plates containing 0.5 per cent yeast extract and 1 per cent glucose. The cells are washed twice by centrifugation and resuspended in 0.033 M of phosphate buffer, pH 5. A yeast suspension prepared in this way will decompose approximately 15 mg. of glucose per hour per 100 mg. of dry yeast under anaerobic conditions at 37°. The initial glucose concentration should not be above 3 per cent.

The mixture of sucrose, yeast, and acid phosphate buffer was centrifuged and the supernatant sugar solution was again passed over the ion exchange columns after the addition of another 100 mg. of sucrose carrier. The neutral fraction was concentrated again and finally reduced to an immobile syrup in the vacuum oven. Upon the addition of warm absolute alcohol

and stirring, the sucrose immediately crystallized. The crystals were placed in a desiccator and after 24 hours filtered off on a sintered glass funnel, washed with absolute alcohol, and dried with ether. The material was then ground in a mortar and assayed for radioactivity. The yield of sucrose was 360 mg, with a specific activity of 0.34 microcurie per mg, giving a total of 122 microcuries.

#### SUMMARY

Methods are described for the isolation of radioactive ( $C^{14}$ ) starch, glucose, fructose, and sucrose from plants exposed to an atmosphere of radioactive carbon dioxide in the presence of light.

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## LETTERS TO THE EDITORS

### THE IN VITRO SYNTHESIS OF HEME IN THE HUMAN RED BLOOD CELL OF SICKLE CELL ANEMIA\*

Sirs

Studies of the red blood cell in a human subject with sickle cell anemia have revealed a random disappearance of heme from the peripheral blood <sup>1</sup> This finding is markedly different from the results obtained in similar studies in normal human subjects <sup>2</sup> This difference may be due either to

200 mg of glycine labeled with 32 atom per cent excess N<sup>15</sup> were incubated at 37° aerobically with 20 ml of heparinized whole blood

Experiment No	Hematologic disorder	Reticulo- cytes	Time of in- cubation	N <sup>15</sup> concentration in hemin N
		per cent	Hrs	atom per cent excess
1	Sickle cell anemia	15	24	0 050
2	" " "	17	24	0 071
3	" " "	20	24	0 071
4	" " "		24	0 077
5	Pernicious anemia*	21	18	0 011
6	Congenital hemolytic jaundice	14	24	0 015
7	" " "	16	24	0 007
8	" " "	11	24	0 013
9	Hypochromic anemia		24	0 020
10	Sickle cell trait		24	0 010
11-16	Normal controls (1 white, 5 Negro subjects)		24	0 000-0 015

\* 100 mg of isotopic glycine used

a random destruction of the red blood cells in sickle cell anemia or to a random synthesis and degradation of hemoglobin in the peripheral blood of the sickle cell anemia subject In the investigation of this problem, the whole blood of subjects with sickle cell anemia was incubated with glycine labeled with N<sup>15</sup> Glycine has been shown to be the nitrogenous precursor of the protoporphyrin of hemoglobin <sup>2 3</sup> The heme isolated as hemin was

\* Aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council

<sup>1</sup> Unpublished data from this laboratory

<sup>2</sup> Shemin, D, and Rittenberg, D, *J Biol Chem*, 166, 627 (1946)

<sup>3</sup> Shemin, D, and Rittenberg, D, *J Biol Chem*, 166, 621 (1946)



found to contain a significant amount of  $N^{15}$ . These results demonstrate an *in vitro* synthesis of heme from glycine by the blood of sickle cell anemia subjects. This is in marked contrast to the findings in control studies with blood from normal subjects and from subjects with other hematologic disorders. These disorders include sickle cell trait without anemia, hypochromic anemia, and conditions, namely pernicious anemia and congenital hemolytic jaundice, associated with reticulocyte counts comparable to those characteristically found in sickle cell anemia.

These data demonstrate that there exists a mechanism in the blood of subjects with sickle cell anemia which can carry out the synthesis of heme *in vitro*. If this mechanism exists in the normal and in the other pathologic states studied so far, it is, under the conditions of these experiments, at best only slightly active. As yet no correlation has been made between the synthesis of heme and the components of sickle cell blood responsible for it. It appears not to be related to the mere presence of numerous reticulocytes.

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# THE IN VITRO SYNTHESIS OF HEME FROM GLYCINE BY THE NUCLEATED RED BLOOD CELL\*

Sirs

It has been demonstrated, in both the rat and human,<sup>1</sup> that glycine is specifically utilized for the synthesis of protoporphyrin of hemoglobin. To facilitate the investigation of the mechanism of porphyrin formation a biological *in vitro* system was sought. Since hemoglobin in the non-nucleated mammalian red blood cell is normally produced prior to the loss of the nucleus, the ability of the nucleated red blood cell from the peripheral

200 mg of glycine labeled with 32 atom per cent excess N<sup>15</sup> were incubated at 37° aerobically with 20 ml of heparinized duck blood and hemin subsequently isolated

Duck No	Time of incubation	N <sup>15</sup> concentration of hemin N
	<i>hrs</i>	
VP-8	24	0.126*
VP-1	12	0.109
VP-2	18	0.303
VP-3	4	0.051
VP-3	18	0.108
VP-3	24	0.113
VP-4	2	0.032
VP-4	6	0.051
VP-4	12	0.088
VP-9	24	0.090
VP-9	24†	0.006

\* This hemin sample was converted to the dimethyl ester of protoporphyrin IX (Grinstein, M, *J Biol Chem*, 167, 515 (1947)). Its N<sup>15</sup> concentration was 0.124 atom per cent excess N<sup>15</sup>. C<sub>2</sub>H<sub>28</sub>O<sub>4</sub>N<sub>4</sub>, calculated N 9.49, found (Dumas) N 9.57

† Incubated at 5°

blood of the duck to synthesize heme *in vitro* was investigated. Incubation of red blood cells of the duck, either in whole blood or in saline, with glycine labeled with N<sup>15</sup> leads to the formation of heme containing N<sup>15</sup>. This demonstrates the utilization of glycine for the synthesis of heme *in vitro*. The data of typical experiments are given in the table.

\* Aided by a grant from the American Cancer Society recommended by the Committee on Growth of the National Research Council

<sup>1</sup> Shemin, D, and Rittenberg, D, *J Biol Chem*, 166, 621, 627 (1946)

This system affords a means to investigate the mechanisms of the synthesis of heme. In addition the use of this *in vitro* system is being extended to the investigation of protein and nucleic acid synthesis.

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# PHOSPHATES OF PYRIDOXAL AND PYRIDOXAMINE AS GROWTH FACTORS FOR LACTIC ACID BACTERIA\*

Sirs

Many cultures of lactic acid bacteria fail to grow in semisynthetic media containing all of the known B vitamins<sup>1</sup> even though oleic acid, recently shown to be essential for growth of many of these organisms,<sup>2</sup> is supplied in the medium

*Comparative Effects of Malt, Pyridoxamine Phosphate, and Pyridoxal Phosphate on Growth of Lactobacillus helveticus\**

Malt (aqueous extract)		Pyridoxal phosphate		Pyridoxamine phosphate	
	Turbidity†		Turbidity†		Turbidity†
mg per 10 cc		γ per 10 cc		γ per 10 cc	
0	95	0	95	0	95
2	87	0 01	78	0 003	81
3	78	0 05	47	0 005	51
4	69	0 10	44	0 01	45
5	57				
10	44				

\* Incubation time, 24 hours at 37°. The basal medium was that previously described (foot-note 2), with yeast extract omitted, and with 50 mg of acid-hydrolyzed, charcoal-treated casein, and 1 γ of pyridoxamine added per 10 cc

† Per cent of incident light transmitted, distilled water = 100

During fractionation of an unidentified factor required for growth of a strain of *Lactobacillus helveticus*, the activity of concentrates was found to be destroyed by light and by incubation with a crude preparation of malt phosphatase. This suggested that a phosphate of one of the light-labile vitamins might be concerned. Direct trial (see the table) showed that synthetic pyridoxal phosphate was highly active. Pyridoxamine phosphate, prepared from pyridoxal phosphate by transamination with glutamic acid,<sup>3</sup>

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<sup>1</sup> Rogosa, M, Tittsler, R P, and Geib, D S, *J Bact*, **54**, 13 (1947)

<sup>2</sup> Williams, W L, Broquist, H P, and Snell, E E, *J Biol Chem*, **170**, 619 (1947)

<sup>3</sup> Rabinowitz, I C, and Snell, E E, *J Biol Chem*, **169**, 643 (1947)

was 3 to 5 times as active as pyridoxal phosphate, and was 1 million times more active than malt, a natural source of the factor. Pyridoxal and pyridoxamine were present in the basal medium, and showed no activity for this organism. In contrast to the vitamin B<sub>6</sub> phosphates, flavin-adenine-dinucleotide and cocarboxylase showed no activity. A preparation of coenzyme II showed 0.003 the activity of pyridoxamine phosphate, coenzyme I was less active. Since these isolated preparations were only 20 and 40 per cent pure, respectively, their slight activity can probably be ascribed to contamination.

The requirement for phosphorylated pyridoxamine or pyridoxal may be quite wide-spread among lactic acid bacteria. A culture of *Lactobacillus acidophilus* also required these substances for growth. For this organism, pyridoxamine phosphate was about 6 times as active as pyridoxal phosphate, and again was 1 million times more active than malt. The amount of pyridoxamine phosphate required for maximum growth of these organisms is closely similar to the amount of pyridoxamine required by organisms able to use the unphosphorylated vitamin.<sup>3</sup>

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# TRACER EXPERIMENTS ON THE MECHANISM OF GLYCINE FERMENTATION BY DIPLOCOCCUS GLYCINOPHILUS\*

Sirs

Glycine is decomposed anaerobically by *Diplococcus glycinophilus*<sup>1</sup> under appropriate conditions according to the equation  $4\text{CH}_2\text{NH}_2\text{COOH} + 2\text{H}_2\text{O} \rightarrow 4\text{NH}_3 + 3\text{CH}_3\text{COOH} + 2\text{CO}_2$ . The mechanism of this fermentation has been investigated by the use of  $\text{C}^{14}$ -labeled glycine, carbon dioxide, and acetic acid. The results of several experiments are summarized in the table.

The data support the following conclusions (1) Approximately 75 per cent of the methyl carbon and 54 per cent of the carboxyl carbon of acetic

*Glycine Fermentations with  $\text{C}^{14}$ -Labeled Substrates*

Ex peri ment No	Substrates	Specific activity, counts per min per mm				Per cent recovery of C <sup>14</sup>
		Labeled sub- strate	Products			
			CO <sub>2</sub>	HAc CH <sub>3</sub>	HAc COOH	
1	C <sup>*</sup> H.NH <sub>2</sub> COOH + NaHCO <sub>2</sub>	12,600	160	9,400	6,800	91
2	CH <sub>2</sub> NH <sub>2</sub> C <sup>*</sup> OOH	17,200	15,500	2,450	8,200	96
3	CH <sub>2</sub> NH <sub>2</sub> COOH + NaHC <sup>*</sup> O <sub>2</sub>	27,600	14,500	1,260	7,460	99
4	CH <sub>2</sub> NH <sub>2</sub> COOH + CH <sub>2</sub> C <sup>*</sup> OONa	1.5 × 10 <sup>6</sup>	180	150	18,200	116
5	CH <sub>2</sub> NH <sub>2</sub> COOH + C <sup>*</sup> H <sub>2</sub> COONa	4.5 × 10 <sup>6</sup>	85	12,100	160	70

\* Washed cell suspensions of *D. glycinophilus* were allowed to act under anaerobic conditions for 16 to 20 hours at 37° on a medium containing approximately 0.04 M glycine, 0.02 M phosphate buffer, pH 7.0, and 0.02 per cent Na<sub>2</sub>S<sub>9</sub>H<sub>2</sub>O. When bicarbonate was added, the initial concentration was from 0.11 to 0.15 M. Acetate was added to Experiments 4 and 5 in tracer amounts ( $<5 \times 10^{-4}$  M).

acid are derived from the methylene carbon of glycine (Experiment 1) (2) From 90 to 95 per cent of the carbon dioxide is derived from the carboxyl carbon of glycine, the remainder coming from the methylene carbon (Experiments 1 and 2). These observations indicate that one of the main reactions in the fermentation is a condensation between 2 molecules of glycine, or derivatives thereof, through their methylene groups. The terminal carbon atoms of the resulting compound, possibly a  $\text{C}_4$ -dicarboxylic acid, are converted mainly to carbon dioxide, and the 2 central carbon atoms are oxidized to acetic acid. The low specific activity of the carbon dioxide

\* Supported in part by a research grant from the United States Public Health Service.

<sup>1</sup> Cardon, B. P., and Barker, H. A., *Arch. Biochem.*, 12, 165 (1947).

formed from methylene-labeled glycine (Experiment 1) shows that a complete oxidation of glycine can occur only to a small extent (3) At least 6 per cent of the methyl carbon and 38 per cent of the carboxyl carbon of acetate originate from carbon dioxide (Experiment 3) A comparison of the specific activities of the acetate carboxyl carbon in Experiments 2 and 3 indicates that a direct reduction of glycine to acetic acid is not an important reaction (4) Acetate is metabolized very slowly if at all (Experiments 4 and 5) This eliminates the possibility that carbon dioxide fixation in this organism involves the reactions  $\text{CH}_3\text{COOH} \xrightarrow{+\text{CO}_2} \text{CH}_3\text{COCOOH} \xrightarrow{+\text{CO}_2} \text{COOHCH}_2\text{COCOOH} \xrightarrow{+6\text{H}} 2\text{CH}_3\text{COOH}$  which would result in a redistribution of the labeled carbon in acetic acid

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# THE RÔLE OF PANTOTHENIC ACID IN THE METABOLISM OF PYRUVATE BY *PROTEUS MORGANII*

Sirs

It has been shown by Dorfman, Berkman, and Koser<sup>1</sup> and by Hills<sup>2</sup> that pantothenic acid is concerned in the oxidation of pyruvate by *Proteus morganii*. This conclusion was based on the stimulation of oxygen uptake by added pantothenate when cells grown in a medium deficient in pantothenic acid were used.

Recently, Novelli and Lipmann<sup>3</sup> have shown that this stimulation is paralleled by an increase in concentration of coenzyme A. Since coenzyme A is necessary for the acetylation of sulfanilamide, these authors have suggested that the pantothenate effect is concerned with the primary attack on acetate.<sup>4</sup>

Experiment No	No pantothenate			0.1 mg. Ca pantothenate		
	O <sub>2</sub>	CO	R Q	O	CO <sub>2</sub>	R Q
	microliters	microliters		microliters	microliters	
1	25	134	5.4	217	600	2.8
2	46	158	3.5	262	386	1.5
3	46	147	3.1	249	508	2.0
Average	39	146	3.7	243	498	2.0

Balance studies performed in this laboratory indicate that the stimulation by pantothenic acid of oxygen uptake of *Proteus morganii* with pyruvate as substrate cannot be accounted for by oxidation of the acetate. The table shows the relation between the oxygen absorption and CO<sub>2</sub> output of deficient cells with and without added pantothenate.

The high R Q of the unstimulated reaction indicates that a decarboxylation takes place but that the oxidation to acetate depends upon an adequate supply of pantothenic acid.

Further investigation has shown that a compound giving a positive Voges-Proskauer reaction accumulates in the reaction mixture when pantothenate has not been added. This compound has been identified as acetyl-methylcarbinol by the formation of nickel dimethylglyoxime after oxidation with ferric chloride.

<sup>1</sup> Dorfman, A., Berkman, S., and Koser, S. A., *J. Biol. Chem.*, **144**, 393 (1942).

<sup>2</sup> Hills, G. M., *Biochem. J.*, **37**, 418 (1943).

<sup>3</sup> Novelli, G. D., and Lipmann, F., *Arch. Biochem.*, **14**, 23 (1947).

<sup>4</sup> Novelli, G. D., and Lipmann, F., *J. Biol. Chem.*, **171**, 833 (1947).



The high  $\eta$  and the accumulation of acetylmethylcarbinol in the unstimulated reaction are not easily explained if the primary attack of the pantothenate enzyme is an acetate. These data suggest that a pantothenic acid containing coenzyme is concerned with the utilization of acetylmethylcarbinol or some closely related substance.

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# REVERSIBLE INHIBITION OF THE COUPLING BETWEEN PHOSPHORYLATION AND OXIDATION

Sirs

Clifton<sup>1</sup> was among the first to show that dinitrophenol (DNP) in low concentrations completely blocked synthetic reactions without interfering with oxidation. Other workers have shown that this drug inhibits nitrogen assimilation,<sup>2</sup> growth and differentiation,<sup>3</sup> the formation of adaptive enzymes,<sup>4</sup> and Hotchkiss<sup>5</sup> has reported preliminary data showing that DNP prevents phosphate uptake by respiring yeast cells. These results would appear to indicate that DNP acts on the basic mechanism in the cell by which phosphate bond generation is coupled to oxidative reactions.

TABLE I

All samples contained 1.0 cc. of an enzyme preparation similar to that of Green *et al.*,<sup>6</sup> prepared by centrifuging a rabbit kidney homogenate in KCl-NaHCO<sub>3</sub> buffer and washing the residue twice with fresh buffer. To this was added 0.1 cc. of yeast hexokinase, 0.0067 M MgCl<sub>2</sub>, 0.013 M NaF, 0.00067 M adenosine 5-phosphate, 0.02 M phosphate buffer of pH 7.2, 0.0167 M fructose, and 0.01 M Na glutamate as substrate. Identical control cups were prepared, into which acid from a side arm was tipped at the beginning of the experiment to provide the initial level of inorganic phosphate. Temperature, 25°, gas phase, air, time, 6 minutes.

Additions	Oxygen uptake	Phosphate uptake	P/O ratio
	microatoms	micromoles	
None	8.0	17.5	2.2
$1.8 \times 10^{-4}$ M DNP	7.9	1.3	0.2

During a study of this coupling mechanism, it was observed that  $5 \times 10^{-5}$  to  $2 \times 10^{-4}$  M DNP prevented phosphorylation without affecting or with slightly stimulating oxidation.

Concentrations of DNP as low as  $5 \times 10^{-6}$  M were found to lower markedly the P/O ratio, an effect that could be reversed by washing out the DNP with fresh buffer. Furthermore, it was found that DNP could "replace" inorganic phosphate, which otherwise is a compulsory component

<sup>1</sup> Clifton, C. E., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, 6, 269 (1946).

<sup>2</sup> Winzler, R. J., Burk, D., and du Vigneaud, V., *Arch. Biochem.*, 5, 25 (1944).

<sup>3</sup> Clowes, G. H. A., and Krahle, M. E., *J. Gen. Physiol.*, 20, 145 (1936).

<sup>4</sup> Spiegelman, S., *J. Cell and Comp. Physiol.*, 30, 315 (1947).

<sup>5</sup> Hotchkiss, R. D., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, 4, 153 (1944).

<sup>6</sup> Green, D. E., Loomis, W. F., and Auerbach, V. H., *J. Biol. Chem.*, 172, 389 (1948).

of this system. It appears that the phosphate-deficient system is strongly stimulated by DNP, while the complete system responds only with a slight stimulation (see Table II).

These results indicate that DNP reversibly uncouples phosphorylation from oxidation, an effect that can also be obtained with atebuin (mepacrine) in  $10^{-3}$  M concentration. Although sodium azide can lower the P/O ratio, it cannot replace phosphate in the system and is, in slightly

TABLE II

Temperature, 25°, gas phase, air, time, 30 minutes

Phosphate, M DNP, M	0 0	$0.8 \times 10^{-2}$ $8 \times 10^{-3}$	$2 \times 10^{-2}$ 0	$2 \times 10^{-2}$ $8 \times 10^{-3}$
O <sub>2</sub> , micromoles	5.1	17.9	18.6	21.0

higher concentration, a powerful inhibitor of respiration as well. DNP does not inhibit respiration except in high concentration.

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# GLYCINE CONTENT OF DL-ALANINE

Sirs

In attempts to employ the assay method for glycine,<sup>1</sup> consistently high blanks were obtained which vitiated assay results. Subsequent receipt of a relatively pure grade of DL-alanine permitted the determination of the amount of glycine in all of our amino acids. Only DL-alanine and arginine yielded results which suggested that they were contaminated with glycine. However, the presence of glycine in arginine seems to be apparent rather than real, this effect is being investigated in this laboratory.

*Glycine Content of Alanine*

Lot No	Source of DL-alanine	Mean per cent glycine
1	A	0.20
2	" , recrystallized 3 times	0.06*
3	" " 6 "	0.04*
4	" " 9 "	0.03*
5	B, Lot 1	1.05
6	" " 2	0.39
7	" " 3	0.39
8	" " 4	0.16
9	" " 5	0.99
10	" " 6	1.86
11	C	0.21
12	D	0.35
13	E	2.7
14	D, $\beta$ -alanine	<0.05

\* Approximate

In the glycine assay,<sup>1</sup> 8 mg of DL-alanine are employed per 2 ml of total volume. The induction period permits 6  $\gamma$  or 0.08 per cent of glycine in the alanine without increase in blank titration. This amount may be further increased by the use of a more dilute inoculum, a practice which the authors<sup>1</sup> do not recommend. We report herewith the glycine content of DL-alanines commercially available (see the table).

Ten commercial lots of DL-alanine, three lots recrystallized three, six, and nine times, respectively, and one lot of  $\beta$ -alanine were assayed by the microbiologic method<sup>1</sup> for their glycine content. Five assay levels were used per sample. Decreasing assay values with increasing amount of DL-alanine were found consistently when the top assay level contained more

<sup>1</sup> Shankman, S., Camien, M. N., and Dunn, M. S., *J. Biol. Chem.*, 168, 51 (1947)

than 4 mg of added DL-alanine per 2 ml of final volume. This decrease is approximately linear with added DL-alanine. Since the value reported is a mean value, it represents a minimal amount of glycine in these samples.

Henderson and Snell<sup>2</sup> have reported a medium containing 0.4 mg of DL-alanine per 2 ml of final volume in which they find one recrystallization of alanine satisfactory. At this level of alanine only 4  $\gamma$  of glycine are introduced at 1 per cent glycine impurity level. Whether this is significant is not possible to say with the brief description given.<sup>2</sup>

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<sup>2</sup> Henderson, L. M., and Snell, E. E., *J. Biol. Chem.*, **172**, 15 (1948).

# THE EFFECT OF PYRUVATE AND INSULIN ON FATTY ACID SYNTHESIS *IN VITRO*\*

Sirs

The utilization of acetic acid for the synthesis of both cholesterol and fatty acids by intact animals has been demonstrated with the aid of labeled acetate<sup>1</sup> In rat liver slices acetate carbon is readily incorporated into cholesterol, but under these conditions the uptake of isotope by the higher fatty acids is very small<sup>2</sup> In order to investigate further the *in vitro*

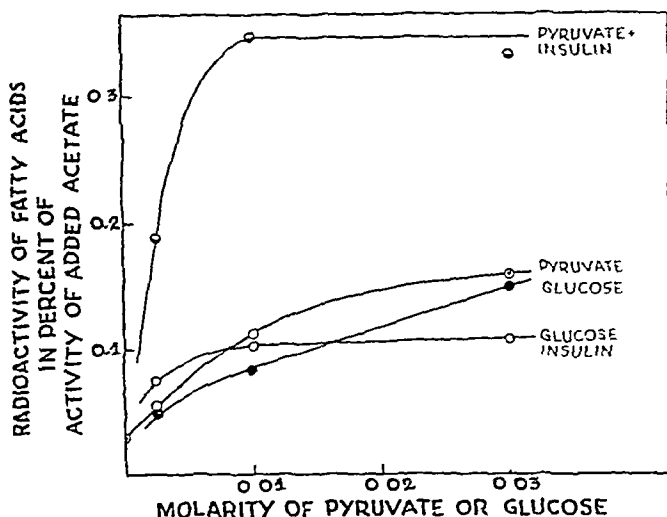


FIG 1 Formation of labeled fatty acids in rat liver slices 1 gm of rat liver slices incubated in 16 ml of Krebs-bicarbonate buffer, pH 7.4, in each vessel for 3 hours at 37°. Gas phase O<sub>2</sub>, all flasks contained 10 mg of C<sup>14</sup> acetate Insulin (letin, Lilly) 0.8 unit per ml

synthesis of fatty acids, rat liver slices were incubated in Krebs-bicarbonate buffer which contained, in addition to CH<sub>3</sub>C<sup>14</sup>OONa, one of the following non-isotopic compounds glucose, pyruvate, oxalacetate, fumarate, malate, or succinate The incorporation of acetate carbon into liver fatty acids was increased several fold by pyruvate and to a somewhat smaller extent by oxalacetate or glucose The other dicarboxylic acids were ineffective

\* Aided in part by a grant from the Dr Wallace C and Clara A Abbott Memorial Fund of the University of Chicago

<sup>1</sup> Bloch, K, and Rittenberg, D, *J Biol Chem*, **143**, 297 (1942) Rittenberg, D, and Bloch, K, *J Biol Chem*, **154**, 311 (1944)

<sup>2</sup> Bloch, K, Borek, E, and Rittenberg, D, *J Biol Chem*, **162**, 441 (1945)

Addition of insulin to the medium afforded a further increase of fatty acid synthesis when the medium contained pyruvate but had no additional effect when glucose was present (see the graph). When acetate was the only substrate, insulin depressed the uptake of isotope by the fatty acids. The stimulatory effect was shown by both crystalline zinc insulin and by an amorphous preparation. A rôle for insulin in the conversion of carbohydrate to fat has been suggested by Drury<sup>3</sup> and emphasized by Stetten and Klein<sup>4</sup> who observed a depressed rate of fatty acid turnover in rats treated with alloxan. The data presented here suggest that one of the actions of insulin is concerned with the metabolism of pyruvate in general<sup>5</sup> and specifically with the utilization of pyruvate for the synthesis of fatty acids.

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<sup>3</sup> Drury, D. R., *Am. J. Physiol.*, **131**, 536 (1940).

<sup>4</sup> Stetten, D., and Klein, B., *J. Biol. Chem.*, **159**, 593 (1945).

<sup>5</sup> Rice, L., and Evans, E. A., Jr., *Science*, **97**, 470 (1943).

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